Nutrition and Serum Protein Levels in Germfree Guinea Pigs[1]

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Gustafsson and Laurell ('58) observed somewhat higher levels of serum albumin in germfree rats than in conventional animals maintained with the same diet, but noted significantly lower beta- and gamma-globulin levels in the germfree animals. Total serum protein levels of the two groups were fairly similar although the mean for the germfree animals was a little less than that for the conventional ones, approximately 5.5 and 5.9 gm per 100 ml, respectively. Recently, Wostmann and Gordon ('60) reported total protein levels which were essentially the same for germfree rats as those in conventional animals. But the germfree animals had proportionately higher levels of albumin than the conventional animals, along with significantly lower levels of the various globulins with the exception of a fraction referred to as alpha-1. In chickens (Wostmann, '59), the principal difference observed between germfree and conventional animals was a reduced gamma-globulin level in the former. This difference was more marked in older than in younger animals.

There is little published information regarding serum proteins in germfree guinea pigs. Miyakawa ('55) reported on 4 germfree animals ranging in age from 20 to 150 days. He observed lower gamma-globulin levels in germfree than in conventional animals, although all the values seemed unusually high. Total protein figures ranged from 5.9 to 6.7 gm with 11 to 13.7% recorded as gamma-globulin, in the germfree; whereas, values for conventional animals ranged from 4.8 to 5.8 gm and 13.1 to 19.4% total protein and gamma-globulin, respectively. As the author points out, the paucity of data warrants little in the way of conclusion. Wostmann ('59) reported on 4 one-year-old guinea pigs shortly after they had become contaminated. Although no numerical values were presented, it was reported that the "slower moving (gamma) globulin fractions" were entirely missing.

On the basis of these limited observations on guinea pigs and the experience with other species, we might have anticipated lowered gamma-globulin values in germfree guinea pigs. It was essential, however, to determine the range of these values as a baseline for a serologic study which we were conducting in connection with induced infections in these animals. During the course of this study, certain interesting relationships were noted between the absence of a flora and the general nutritional state of the germfree guinea pig, as gauged by weight gain and serum protein levels.

MATERIALS AND METHODS

Germfree guinea pigs (GF) were obtained by Caesarean section of conventional pregnant females from the National Institutes of Health "hybrid" colony, and were maintained in Reyniers Germfree System units, essentially according to procedures and sterility checks described previously (Reyniers et al., '46; Phillips et al., '59). Periodically, samples of fresh feces, food, bedding, et cetera, were examined microscopically and inoculated into various media. Failure to demonstrate the presence of viable organisms constituted the basis for calling the animals "germfree." A modification of diet L-445, recommended by Phillips et al. ('59), was used. The ani-

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mals were offered all they would eat within about an hour, twice a day, as these authors recommend. Diet L-445 is steam-sterilized and is, for the most part, an equal mixture of ground commercial laboratory chow[4] and rolled oats, with vitamin supplements. This diet, with some modifications, has been the one most commonly used for rearing germfree guinea pigs, as nearly as can be determined from the sparse literature on this subject. Our modification has been a substitution of the stock commercial guinea pig pellets[5] for commercial laboratory chow,[6] and the addition of kale and carrots (Warren and Newton, '59). In addition, some of the supplements (ascorbic acid and thiamine) are sterilized by filtration rather than by steam.

Each unit contained the litters from three females, usually a total of 9 to 10 guinea pigs. In one series of comparisons, animals from each litter were removed from a unit at the ages of one, two, and three weeks. In another series, involving older animals, all the animals were removed from the unit at the age of 12 weeks. Several blood samples were taken from each animal by puncturing the heart and allowing the blood to flow into small melting-point capillary tubes approximately 1.5 × 100 mm. The serum was separated from the clot and loose red cells by centrifugation and frozen until used. When the blood was obtained in this manner hemolysis rarely occurred; any sample showing hemolysis was discarded.

Two groups of conventional animals obtained from the same guinea pig colony at the age of two to 4 days were used for comparison and were maintained in an ordinary animal room. One group (CV) was fed, ad libitum, the usual nonsterilized stock diet of commercial guinea pig pellets supplemented with kale and carrots. As a control for the germfree animals, the other group (CVD) was maintained with the same autoclaved ration given the germfree animals. The food was sealed immediately after sterilization and kept refrigerated in small containers until given to the animals. The CVD animals were also offered all they would consume in about an hour, twice a day. This feeding schedule was adopted in an effort to duplicate the food consumption and eating habits of the germfree animals.

Aside from containing living microorganisms, the controls also differed from the germfree animals in that they were delivered normally and had suckled for a few days before being weaned. As has been observed before (Phillips et al., '59; Newton et al., '60), however, these variables have been difficult to control because it has not been possible to keep Caesarean-born, nonsuckled guinea pigs alive for appreciable periods with any consistency outside the germfree system.

The serum protein determinations were carried out on a Spinco Model R paper electrophoresis apparatus according to standard techniques. Schleicher and Schuell 2043A-mgl paper strips and a barbiturate buffer solution (0.1 N, pH 8.6) were used. Migration of the protein components was induced by applying a constant current of 5 ma for 16 hours. Staining was with bromphenol blue and evaluation was made spectrophotometrically with the Model RB Analytrol, fitted with a B-5 cam.

Four fractions were identified on the stained strips and in the densitometer scannings. In order of decreasing mobility, they will be referred to as albumin, alpha-globulins, beta-globulins, and gamma-globulins, respectively. The mobilities of these fractions were similar to those of comparable fractions of human serum.

Total serum protein concentrations were determined spectrophotometrically using the ultramicro technique of Caraway and Fanger ('55).

RESULTS AND DISCUSSION

In figure 1 are presented a typical strip and analysis of a serum sample from one of the older conventional guinea pigs fed the ordinary stock diet. The 4 fractions which we felt we could identify consistently are indicated, and form the framework for calculating the values obtained. Serum protein determinations were performed on a total of over 100 animals in the three groups, and the results are shown

[4] Purina Laboratory Chow, The Ralston-Purina Company, St. Louis.
[5] Sherwood Mills, Baltimore Feed and Grain Company, Baltimore, Maryland.
[6] See footnote 4.

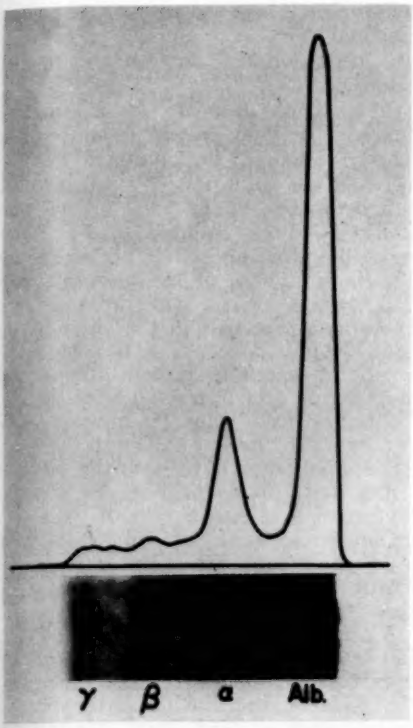

Fig. 1 Typical electrophoretic pattern of serum from a 12-week-old conventional guinea pig.

in figure 2. The values for the two-week-old animals were similar to those of the three-week-old group and have been omitted to facilitate comparisons. Each bar represents an average based on 9 to 10 animals. The data were analyzed statistically by the analysis of variance method. An arrow connecting two bars indicates that there was a significant difference between these average serum protein values at the 1% level. Data were analyzed for "sterilized diet effect" among conventional animals (CV vs. CVD) and "germfree effect" (CVD vs. GF). Differences occurring between CV and GF were not considered for purposes of comparison inasmuch as these groups differed in at least two variables.

Turning first to the analysis for possible sterilized diet effect, there was no significant difference in the levels of total protein, and of most of the individual serum components, between the CV and the CVD. There was a slight gradual increase in both groups, up through the age period studied. Some differences were observed in gammaglobulin levels, however. The levels in the one-week-old animals were the highest. Presumably this was a reflection of transplacental transmission. In the two- and three-week-old animals the levels had dropped in both groups, more markedly in the CV than in the CVD. In the 12-week-old animals, the gamma-globulin levels of the CV animals showed an increase over those of the three-week-old group, whereas those of the CVD group continued the downward trend. Although the numerical values were

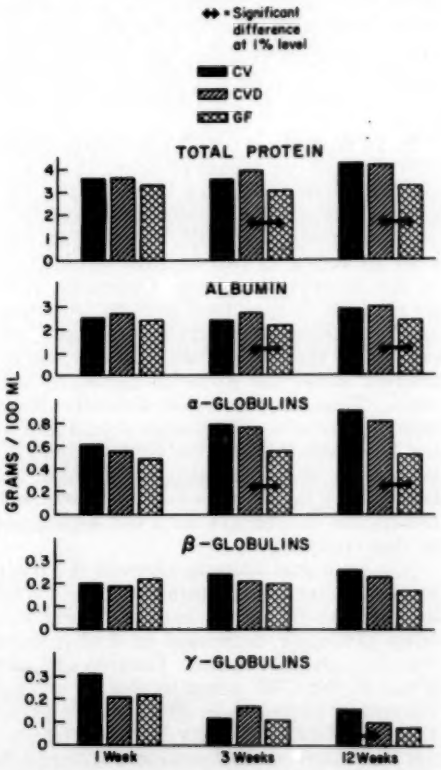

Fig. 2 Mean serum protein values of guinea pigs of different ages. CV indicates conventional animals fed stock diet; CVD, conventional animals fed germfree type diet; GF, germfree animals. Statistical analysis between CV vs. CVD, and CVD vs. GF, only.

low in both groups, this difference proved to be significant at the 1% level.

Whether such low levels would persist in older conventional (contaminated) guinea pigs fed the sterilized diet remains to be seen. On the basis of the findings with these 12-week-old animals, however, one can conclude that sterilization of the diet was a factor contributing to the maintenance of lower gamma-globulin levels in these otherwise "contaminated" animals. As mentioned earlier, the food for the CVD animals was kept sterile until offered to them, and was consumed rather quickly. This undoubtedly reduced to a considerable extent both the numbers, and perhaps the kinds, of living organisms ingested by these animals. This, in turn, could have had an effect on the stimulation of gamma-globulin production. This apparent relationship must be explored further because of its interesting implications.

In an analysis of possible germfree effect (CVD vs. GF) on serum proteins, there was essentially no difference between the two groups among the one-week-old animals. Such an effect was noted, however, in animals as young as two to three weeks, and in the 12-week-old group. Generally, with an increase in age, total protein, albumin, and alpha-globulin levels gradually increased in the CVD, whereas the levels remained about the same in germfree animals. Thus, in the oldest animals, there were differences, which were significant at the 1% level, between the GF and the CVD groups in these proteins. Although the beta-globulins also appeared to show these trends, the differences were not significant in this study.

The youngest animals showed the highest values for the gamma-globulins. With an increase in age of animals, these proteins gradually decreased in both groups up through 12 weeks. Gamma-globulin levels in the CVD group tended to average somewhat higher than those in the germfree, but not significantly so. As mentioned earlier, there might be greater differences between CVD and germfree guinea pigs in older animals, as observed in chickens by Wostmann ('59). In animals 12 weeks of age, however, we were unable to demonstrate a significant difference in gamma-globulin levels between animals without an intestinal flora and animals maintained in a "contaminated" environment but fed a sterilized diet.

Of particular interest was the marked difference in total protein levels between the conventional ("contaminated") guinea pigs and the germfree. Values obtained with both the CV and CVD groups were similar to those reported in the rather sparse literature on guinea pig serum protein levels. Those of the germfree were 25% lower, however. This difference was represented primarily in the albumin and alpha-globulin portions. It is known that nutrition, especially protein intake, can affect serum protein levels (Albanese, '59; Yoshimura, '61). Thus, the question arose as to whether the low values in the germfree animals may have been the result of a nutritional inadequacy of the diet, an inadequate food intake, or both. In table 1 are presented some data on weight gains and food consumption of the animals comprising the 12-week-old-group, from the third week on.

A comparison of male weights indicates that both groups of the conventional animals reached considerably larger sizes than the germfree, and that the latter were undernourished at least from this point of view. Moreover, results with the CVD animals indicate that the sterilized diet allowed fairly good growth in conventional animals (namely, those with a flora), although not as good as that shown by the animals allowed to eat the stock diet ad libitum (CV) and as reported normal by Reid ('58). Cohn and Joseph ('60) have shown that, although the feeding schedule (ad libitum versus timed meals) may affect fat deposition and other metabolic processes, weight gains are about the same when total quantities of the food ingested are the same. It is likely that had the CVD animals been given more opportunities to feed, approaching an ad libitum schedule, they would have consumed more within a 24-hour period, and their weights might have more closely approximated those of the CV animals. (In fact, in a subsequent study, conventional male guinea pigs which were maintained with this same sterilized diet, but which were allowed to eat more frequently, did consume more

food per day and attained weights of 550 to 600 gm at 12 weeks of age.) Thus, it would appear from both weight gain and total serum protein levels in the CVD group that the nutritional adequacy of the sterilized diet *per se* was not the essential reason for the poorer results obtained with the germfree animals.

The amounts of food consumed could, of course, have been a factor. While both the germfree and the CVD animals were consuming about the same amount of food at mealtimes at the start, as they became older it was apparent that the CVD animals could eat gradually more than the germfree. Thus, records of the approximate amounts eaten by both the germfree and the CVD groups were kept after the 5th week. Average daily dry-weight food consumption per animal during the 11th week is shown in table 1. These figures include both sexes, with about an equal distribution in each group. The CVD animals ate 35% more than the germfree during the 11th week. When average food consumption is related to average body weight, however, the two groups were eating at about the same rate. Each was consuming approximately 6% of its body weight per day at this time. This was somewhat less than what has been estimated as normal for guinea pigs fed a stock-type diet ad libitum—8.3% of body weight per day, according to Reid ('58)—and may account in part for weights lower than those of the CV group, as discussed previously. It would appear, however, that weight differences between the CVD and the germfree animals cannot be explained by differences in food consumption.

Further analysis of the food consumption emphasizes this point, as shown by the figures at the bottom of the table. At necropsy at 12 weeks, approximately one-third of the total body weight of the germfree animal consisted of cecum and contents (primarily the latter), as compared with much lower figures for the other animals. The large cecum, of course, has been observed by all who have worked with the germfree animal. These values point up the fact that the germfree animals were actually consuming more food in relation to overall carcass size than were the CVD animals. The cecum and contents of the latter tended to weigh a little more than those of the animals receiving the unsterilized stock diet. The differences were small, however, and it is felt that a study involving more animals would be required to establish the validity of such a difference.

Observations on the mortality of the germfree animals are of limited value inasmuch as most were sacrificed at a fairly early age. There was a loss of approxi-

TABLE 1

Comparative weight gains, food consumption and cecal size of germfree and conventional guinea pigs

Age in weeks	Germfree (GF)	Conventional, fed "germfree" diet (CVD)	Conventional, fed stock diet (CV)
	Average weight,[1] gm		
3	154	209	236
6	233	270	337
9	277	377	442
12	337	459	577
	Average daily food consumption,[2] gm dry weight		
11–12	20 (6)[3]	27 (6)	ad libitum (8)[4]
	Average weight of cecum and contents/total body weight[2]		
12	32% (25–37%)	7% (6–10%)	4–5%

[1] Males only, 5 animals.
[2] Both sexes, 9 to 10 animals.
[3] Figures in parentheses indicate percentage of body weight.
[4] According to Reid ('58).

mately 15%. Most of the deaths occurred within the first week following Caesarean delivery, and in the larger litters.

In a comparison of the weights of the germfree guinea pigs in this study with those obtained by Phillips et al. ('59) with diet L-445, 10 germfree animals, presumably of mixed sexes, weighed an average of 133 gm at 20 days in their study; a similar group averaged 151 gm at 21 days in our study. At the ages of 30 days and 28 days, the average weights were 160 and 174 gm, in their and our groups, respectively. Beyond this age there are not enough data suitable for comparison. While the body weights in the present study tended to run a little higher than in that of Phillips et al., they were reasonably similar, and in both studies were below what are considered normal for the conventional (contaminated) animals.

It would seem, therefore, that the lack of a flora contributed in a large measure to the substandard nutrition of the germfree guinea pigs. This inadequate nutrition may, in turn, have affected serum protein levels. Perhaps with the crude diet (grain-grass-vegetable mixtures), bacteria are needed to perform digestive or other enzymatic functions which provide the guinea pig with certain important nutritive elements. Some of these may not even have been determined as yet, inasmuch as most of the information on the nutritional requirements of the guinea pig has been obtained on animals with a flora. Horton and Hickey ('61), using an irradiation-sterilized semisynthetic diet supplemented with amino acids, vitamins and minerals, appeared to be getting a little better growth in germfree guinea pigs. But even with this highly fortified diet, growth of the germfree animals was still only about 75% of that of conventional (contaminated) animals receiving the same sterilized diet, at 8 weeks.

The unusually enlarged, thin-walled cecum must be considered also. This area of the intestine appears to be a comparatively important one in the guinea pig (Hagen and Robinson, '53), and abnormalities could interfere with proper absorption of critical elements.

SUMMARY

Serum protein levels were compared, by paper electrophoresis, in germfree and conventionally-reared guinea pigs up to the age of 12 weeks. The animals were maintained with a crude diet of guinea pig pellets, rolled oats, kale and carrots, both steam-sterilized and unsterilized. Weights and food consumption were recorded during the growth of the older animals.

Gamma-globulin levels in 12-week-old germfree guinea pigs were similar to those in conventional animals maintained with the same sterilized diet; however, levels in both groups were below those in conventional animals maintained with the unsterilized diet. Germfree animals had 25% lower total serum protein levels than conventional animals fed the same sterilized diet. Most of the difference was in the albumin and alpha-globulin levels.

Germfree guinea pigs did not grow as well as conventional animals fed the same sterilized diet on the same feeding schedule. Weights averaged only 74% of those of the conventional animals at 12 weeks; in addition, 25 to 37% of the weights of the germfree guinea pigs consisted of cecum and contents, as compared with an average of 7% in the conventional animals. Calculations indicated that the germfree animals actually had a higher daily food consumption on a body-weight basis, excluding the weight of the cecum and contents, than did the conventional animals.

It would appear, therefore, that the lack of a bacterial flora affected adversely the nutrition of germfree guinea pigs fed a crude diet. The lower total serum protein levels could have been a reflection of this inadequate nutrition.

ACKNOWLEDGMENT

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Caloric Requirements of Man in the Antarctic

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A perusal of the nutritional literature indicates that the effects of low environmental temperatures per se on the caloric requirements of adequately clothed men in the Arctic are small when compared with the greater effects of increased physical activity, the hobbling effects of cold weather clothing and the increased energy cost of walking through snow (Gray et al., '51; Rodahl, '58; Iampietro et al., '58). Rodahl's ('54) review of nutritional studies in the Arctic shows that these caloric requirements do not deviate materially from those of men of similar body size, engaged in comparable activities in more temperate climates. This conclusion is supported by more recent studies (Welch et al., '58; Masterton et al., '58; Ponomarev and Sokolova, '59).

Although one should expect a similar situation in the Antarctic, little specific information is available pertaining to caloric requirements in this region where climatic conditions are much more severe than in the Arctic. Available information refers to trail rations, their composition and caloric content, without records of food actually consumed and its effect on body weight (Hayes, '28; Lockhart, '45). In view of the tremendous logistic problems involved in supporting human operations in the Antarctic, and the increased importance of this distant area in human affairs, exact data pertaining to caloric requirements of men on Antarctic service are of considerable practical importance. It is unsound to provide more food than required to maintain normal body weight; overfeeding leads to overweight and the concomitant decrease in physical fitness is undesirable.

The purpose of this paper is to report the results of a series of dietary surveys carried out at Little America V (lat. 78° 11′ S, long. 162° 10′ W) in the Antarctic during 1957. The subjects were International Geophysical Year (IGY) scientists and U. S. Navy sailors who lived at this isolated station for 12 months. The climate of this region is characterized by low environmental temperatures and winds of high velocity; there are 4 months of darkness in winter. Climatic conditions are illustrated graphically in figure 1.

MATERIALS AND METHODS

These surveys included 9 IGY scientists (mean age 33 years, weight 82 kg, height 181 cm) and 4 U. S. Navy personnel (mean age 28 years, weight 81 kg, height 176 cm). Five of the scientists were studied in March (austral fall), 4 in June (winter) and 4 in September (spring). The 4 Navy personnel were studied in September. The dietary surveys lasted for 6, 5, 6 and 5 days, respectively.

The experimental protocol was as follows. Subjects were allowed to select food as usual in unlimited quantities. Each food item was collected in a separate plate or cup and weighed on a dietary scale. At the end of the meal each plate or cup, together with the remaining unconsumed food, was again weighed and the food consumption recorded for each individual separately. Between-meal consumption was recorded on separate sheets by the subjects. For the computation of the results the values of the foodstuffs were taken from Watt and Merrill ('50), Mattice ('50), McCance and Widdowson ('47) and the U. S. War Department TM 8-500 ('45).[2]

Energy expenditure of the subjects was estimated on the basis of time-activity

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[2] Wooster, H. A., and F. C. Blanch 1949 Nutritional Data. Heinz Nutritional Research Division, Mellon Institute, Pittsburgh.

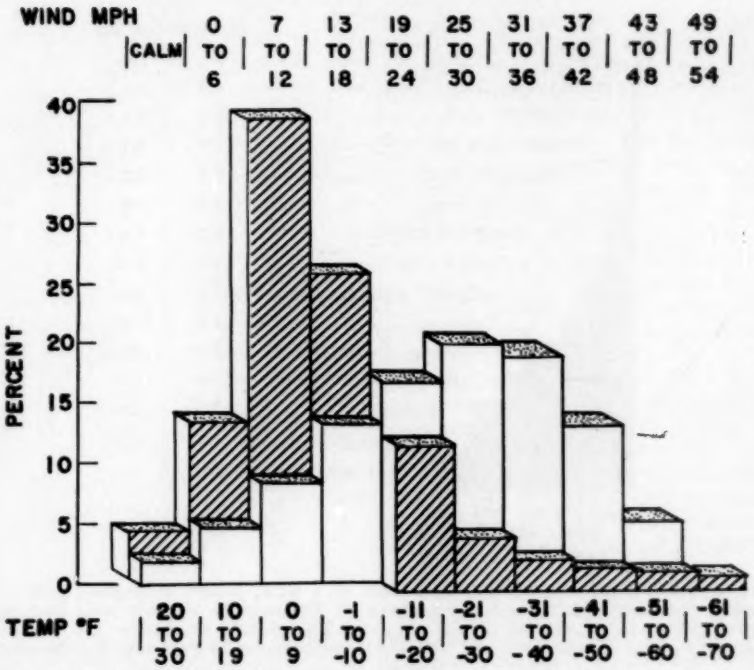

Fig. 1 Frequencies of wind velocities (Hachure) and dry bulb temperatures March to October, 1957, Little America V.

data, as illustrated graphically in figure 2. The figures compiled by Passmore and Durnin ('55) for energy expenditure of various activities were used in these estimations. In addition, the metabolic cost of certain outside activities such as walking at different speeds and on different snow surfaces was measured. Expired air volumes were measured on an insulated and artificially warmed respiration Gasuhr developed by the Max Planck Institute for work physiology (Lehman, '53; Montoye et al., '58), which the subject carried on his back. Aliquot samples of expired air collected in punching bag bladders were analyzed for oxygen content on a Model C Beckman Analyzer. Gas volumes were reduced to standard temperature and pressure-dry, and energy costs of these activities were calculated by the method of Weir ('49).

All members of the expedition lived a regulated existence. Reveille, meal time and "lights out" occurred at set hours.

RESULTS AND DISCUSSION

After two months in the Antarctic, the first survey showed that 5 scientists were expending an average of about 3775 Cal. and consuming 3400 Cal. per day. The results of the winter survey showed that 4 scientists expended an average of 3370 Cal. and consumed 4396 Cal. per day. Over the intervening 4 months these subjects gained an average of 3.7 kg. In the spring 4 scientists expended 4175 Cal. and consumed 4285 Cal. per day. The average weight gain following the previous survey was 1.3 kg.

The scientists spent an average of 13% of the time (range 6 to 19%) working outside in the fall (March, '57). In winter (June, '57) only 6% of the time was spent outside (range, zero to 12%) whereas 15% (range, zero to 36%) of the time was spent outside in the spring (September, '57).

The results of a dietary survey undertaken on 4 sailors in the spring showed

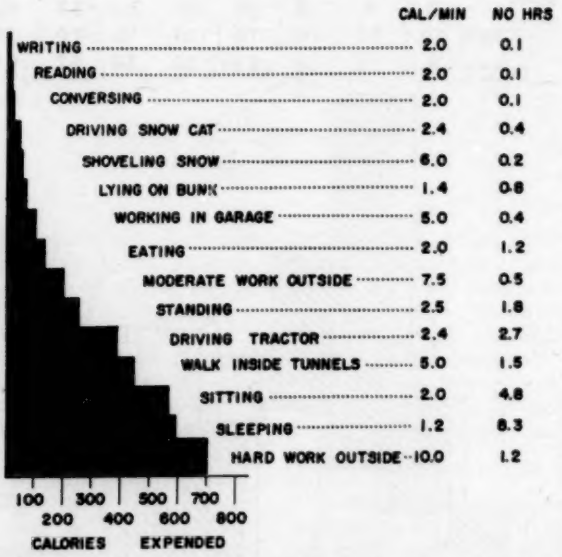

Fig. 2 Average 24-hour energy expenditure of 4 U. S. sailors determined over a 5-day period in the austral spring at Little America V. The number of hours were derived from a time-activity study compiled by the subjects. Energy cost in calories per minute was obtained by actual measurement and from the tables compiled by Passmore and Durnin ('55).

that they expended an average of 3660 Cal. and consumed 4925 Cal. (table 1). The 11 months' average weight gain in these subjects was 4.6 kg. These Navy subjects were acting as support personnel and they averaged 20% of the total time outside (range, 9 to 31%).

The values for the percentages of calories furnished by protein, fat and carbohydrate in the foods were determined by using standard nutritional tables (table 1). These percentages were not significantly different from those in the diet of United States troops eating a garrison ration elsewhere. There was no appreciable increase in fat consumption over the year. About 33 to 37% of the calories in the diet of the scientists were obtained from fat. This is close to the figures for fat intake by infantry soldiers in Alaska (Rodahl, '54) or soldiers eating a garrison ration in temperate or tropical climates (Johnson and Kark, '46; Swain et al., '49). In the sailors' diet 43% of the calories were derived from fat. This, however, is no higher than the figures reported by McCay ('45) for U. S. Navy personnel in a temperate environment (44%) or continental U. S. Army personnel (43%) reported by Schor and Swain ('48), or the 42% reported more recently by Consolazio et al. ('59).

Passmore and Durnin ('55) consider that the estimation of energy expenditure is at least as accurate as the estimation of caloric intake if the time of the activity is accurately determined. They have further concluded that the estimation by dietary survey of the caloric intake of even a relatively homogeneous group of persons is subject to some error, to which the use of tables of food analyses contributes. Woolf ('54) has estimated that a 10% probable error in estimating caloric intake occurs even under the best conditions. Unfortunately, it was not possible to compare analyzed and calculated values in this survey for practical reasons.

These Antarctic rations were provided by the U. S. Navy Subsistence Office. An "average" daily ration weighed 2.1 kg and provided 5944 Cal. Eleven per cent of these calories were derived from protein,

TABLE 1

Mean daily intake of calories, protein, fat and carbohydrate by IGY[1] and Navy personnel in the Antarctic

Subjects	Body weight	Calories	Percentage of calories derived from		
			Protein	Fat	Carbohydrate
	kg				
March, '58, 5 IGY	81.2 (72.3–93.2)	3400 (2908–4181)	14.2 (11.8–15.1)	36.5 (33.2–39.4)	49.2 (45.7–55.0)
June, '58, 4 IGY	83.6 (67.2–93.6)	4396 (3675–5521)	13.2 (10.7–15.8)	32.5 (28.8–36.6)	54.2 (47.6–60.5)
September, '58, 4 IGY	79.7 (67.3–93.2)	4285 (3080–5141)	13.4 (12.2–14.6)	36.2 (33.1–39.6)	50.5 (45.8–54.7)
September, '58, 4 Navy	80.6 (75.4–85.4)	4925 (3724–6847)	14.0 (12.4–15.9)	43.0 (38.5–46.3)	43.0 (37.8–49.1)

[1] International Geophysical Year scientists.

41% from fat and 48% from carbohydrate. Of the approximate 73 Cal. per kg of body weight per day theoretically provided in the mess, between 41 and 53 Cal. per kg were consumed by the scientists and 61 Cal. per kg by the sailors. The energy requirement of the scientists ranged between about 41 and 51 Cal. per kg and that of the sailors was approximately 45 Cal. per kg. Consequently all subjects gained weight on this dietary regimen. It may therefore be concluded that the diet supplied to the Antarctic personnel was more than adequate in total calories to meet requirements.

Brozek and Mickelsen ('49) have discussed the important psychological aspects of overeating in the total behavior of submarine personnel confined to cramped quarters under trying circumstances for long periods. It appears that psychological factors may play a similar role among overwintering personnel in the Antarctic.

SUMMARY AND CONCLUSIONS

Dietary surveys and estimates of energy expenditure were made in a small but representative sample of International Geophysical Year (IGY) scientists and Navy personnel at Little America V in the Antarctic during the International Geophysical Year, 1957–'58. For the IGY personnel the average daily expenditure was about 3775 Cal. and the consumption 3400 Cal. per man in March, 3370 and 4396, respectively, in June, and 4175 and 4285, respectively, in September. The Navy personnel expended 3660 Cal. and consumed 4925 Cal. per day in September. On this regimen all of the subjects gained weight during the Antarctic stay. It may therefore be assumed that the caloric intakes reported here are, on the whole, in excess of actual caloric requirements under these conditions. The values for the percentages of total calories consumed furnished by protein, fat and carbohydrate are not different from those reported for United States troops eating a garrison ration in Arctic or temperate climates.

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Effect of Dietary Fatty Acids and Cholesterol on Growth and Fatty Acid Composition of the Chicken[1]

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After 6 to 8 weeks the growth of chickens fed diets almost free of unsaturated fatty acids will increase if corn oil is added to the diet (Bieri et al., '56). This suggests that the chicken requires essential fatty acids (EFA). It is not known whether it is the linoleic plus the linolenic acid or linoleic acid alone in the corn oil which results in a growth response, or whether other dietary variables can affect the requirement of the chicken for EFA. Studies with the rat have demonstrated that addition of cholesterol to diets containing fat (Holman and Peifer, '60; Gambel and Quackenbush, '60) will depress growth and accelerate EFA deficiency symptoms. Saturated fats also inhibited growth of EFA deficient rats (Deuel et al., '51; Funch et al., '57; Peifer and Holman, '59). These effects have not been demonstrated in the chicken and have not been adequately explained in the rat. The following experiments were designed to determine whether cholesterol and saturated fatty acids (SFA) affect the onset or development of EFA deficiency disease in the chickens and whether growth differences observed could be rationalized on the basis of alterations in fatty acid composition of critical tissues.

Dam et al. ('56), Dam and Nielsen ('57), and Hølmer et al. ('60) have demonstrated with the alkaline isomerization method that addition of cholesterol to the diet decreased the tetraene content of the liver of chickens. No effects on growth were reported, however. The alkaline isomerization procedure does not differentiate between unsaturated fatty acids of different chain lengths and may be in error because of the arbitrary method of calculation. Therefore in studies reported here the effect of cholesterol on the fatty acid composition of various tissues was re-investigated using gas chromatography. The effect of the addition of saturated and unsaturated fatty acids to fat-free diets on tissue fatty acid composition of growing chickens was also determined.

In the rat linoleic acid is an EFA, whereas the usual isomer of linolenic acid (9,12,15-octadecatrienoic acid) cannot be converted to either linoleic acid or arachidonic acid (Steinberg et al., '57) and is only slightly active (Hume et al., '40; Greenberg et al., '50; Thomasson, '53) as an EFA. Comparable studies have not been conducted with the chicken. Based on fatty acid analysis with the alkaline isomerization procedure Reiser ('50, '51) concluded that the chicken can convert linolenic acid to polyunsaturated fatty acids having from two to 6 double bonds, thus suggesting that linolenic acid may have EFA activity in the chicken. It was therefore of interest to determine whether linoleic or linolenic acid or both can promote growth of chickens fed fat-free diets.

EXPERIMENTAL

The basal diets used in this experiment are given in table 1. The diets contain not more than 0.005% of linoleic acid. Safflower oil was fed as a source of linoleic acid in experiments one and two. Diets were fed ad libitum. Vitamin A was supplied in the drinking water (Bieri, '59) at 10 and 20 days. At the end of 4 weeks, pooled samples of livers from chickens in each treatment were observed to contain 700 to 1600 IU of vitamin A per gm. Therefore, we are confident that the stud-

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[1] A preliminary report has been presented in Federation Proc., *19*, 222, 1960 (abstract).

TABLE 1

Composition of experimental diets

	C-21	C-21-A
	%	%
Vitamin-free casein[1]	21.000	21.000
L-Glutamic acid	5.000	5.000
L-Arginine·HCl	0.600	0.600
MHA[2]	0.200	0.200
Glycine	2.000	2.000
Glycerol	—	0.500
Methyl myristate	1.000	—
Methyl laurate	—	10.000
Salt mixture F-J[3]	5.400	5.400
Vitamin mixture[4]	0.500	0.500
Santoquin[5]	0.015	0.015
Choline chloride	0.200	0.200
Vitamin A concentrate (50,000 IU/gm)	0.050	0.050
d α-Tocopheryl acetate	—	0.010
Vitamin D concentrate (7,500 ICU/gm)	0.020	0.020
Penicillin concentrate (50%)	0.002	0.002
Furazolidone concentrate (22%)[6]	0.050	—
Glucose[7]	q.s. 100	q.s. 100

[1] "Vitamin-Free" Casein, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[2] Registered trademark of the Monsanto Chemical Company for calcium *dl*-2-hydroxy, 4-methylthiobutyrate.

[3] This will supply as percentage of the diet: $Ca_3(PO_4)_2$, 2.8; K_2HPO_4, 0.90; $MgSO_4 \cdot 7H_2O$, 0.25; $Fe(C_6H_5O_7) \cdot 6H_2O$, 0.14; $ZnCO_3$, 0.015; KI, 0.004; $CuSO_4 \cdot 5H_2O$, 0.002; H_3BO_3, 0.0009; $CoSO_4 \cdot 7H_2O$, 0.001; $MnSO_4$, 0.065; NaCl, 0.88; $Na_2SeO_3 \cdot 5H_2O$, 0.0001; cornstarch, 0.0229.

[4] This will supply in milligrams/kilogram of finished diet: vitamin B_{12}, 0.02; biotin, 0.30; menadione, 1.0; pyridoxine·HCl, 8.0; folic acid, 4.0; riboflavin, 16.0; Ca pantothenate, 20.0; thiamine·HCl, 24.0; nicotinic acid, 100.0.

[5] Registered trademark of the Monsanto Chemical Company for the antioxidant, 6-ethoxy, 1,2-dihydro, 2,2,4-trimethyl-quinoline.

[6] NF-180, Hess and Clark, Inc., Ashland, Ohio.

[7] Cerelose, Corn Products Refining Company, New York.

ies were not complicated by a vitamin A deficiency.

All studies were conducted with Nichols 108 cockerels reared in electrically heated, wire-floor cages, maintained in an air-conditioned room provided with continuous illumination. The basal diet was fed to all birds for 6 or 7 days. The birds closest to the mean weight were divided into experimental groups of 12 birds each, replicated three times and fed the experimental diets.

Fatty acid analysis. Chickens were fasted for 14 to 16 hours, killed, and the tissues were dissected and frozen rapidly by pressing them against aluminum foil held against solid carbon dioxide. The tissues of 5 birds from each treatment were pooled and stored at −15°C. The pooled samples were saponified, and the resulting soaps converted to fatty acids which were extracted and converted to methyl esters. The methyl esters were then analyzed by gas chromatography. Details of these procedures have been described previously (Marco et al., '61). The results are expressed as per cent by weight of total fatty acids excluding any with a retention time higher than eicosapentaenoic acid (20:5).[1]

RESULTS

In contrast with previous observations of the chicken, or other animals, a growth response was observed after a very short feeding period (one week with basal diet C-21 and one week with the supplemented diet, table 2). There was a significant growth depression from the addition of SFA to EFA-deficient diets and a further depression at 27 days when cholesterol was added to the diets containing the SFA. Although the effect of cholesterol was not statistically significant in this study, in the following and subsequent

[1] Carbon chain length: number of double bonds.

studies (table 5) a significant depressing effect of cholesterol on growth was observed. Cholesterol had no effect when added alone.

After feeding the chickens basal diet C-21 for 7 days and the supplemented diets for 43 days, several representative tissues were excised and analyzed for fatty acids by gas chromatography. The fatty acid composition of the liver is given in table 3. Addition of 2% of safflower oil markedly increased the concentration of linoleic acid (18:2) and arachidonic acid (20:4) and decreased palmitoleic acid (16:1), oleic acid (18:1) and a C-20 triene (20:3). The addition of a mixture of

TABLE 2

Effect of fatty acids and cholesterol on growth

Supplements to diet C-21			7–14 Days	7–27 Days	
Laurate + added myristate[1]	Cholesterol	Safflower oil[2]	Gain[3]	Gain	Feed conversion
%	%	%	*gm*	*gm*	*gm feed/ gm gain*
0	0	0	62	259	1.91
8	0	0	54	217	2.07
0	2	0	60	252	1.92
8	2	0	51	185	2.06
0	0	2	74	301	1.90
8	0	2	68	285	1.88
0	2	2	70	306	1.81
8	2	2	66	279	1.79

[1] 5% Methyl laurate, 3% methyl myristate added in place of glucose.
[2] Added isocalorically by replacing 3.8% glucose with cellulose in these treatments.
[3] Average weight was 84 gm at 7 days. According to a regression analysis the effect of safflower oil and of the laurate-myristate mixture significantly ($P < 0.01$) affected gain in both the 7 to 14-day and 7 to 27-day periods.

TABLE 3

Effect of diet on fatty acid composition of liver fat[1]

Laurate + myristate[2]	% of the diet							
Cholesterol[2]	0	0	8[3]	8	0	0	8	8
Safflower oil[2]	0	2	0	2	0	2	0	2
	0	0	0	0	2	2	2	2
	% of liver fat							
Fatty acid[4]								
12:0	0.1	0.0	0.2	0.3	0.1	0.2	0.5	0.4
14:0	0.5	0.6	2.4	2.8	0.6	0.5	2.2	2.9
16:0	17.0	16.3	16.1	14.7	21.5	18.4	20.1	17.3
16:1[5]	6.1	18.9	6.3	7.1	0.8	1.7	2.0	1.6
18:0	20.2	6.6	19.9	16.1	27.6	24.1	25.7	26.6
18:1	40.7	42.3	39.3	45.1	12.4	19.9	12.8	18.8
18:2	1.1	0.0	1.1	2.0	16.2	17.3	18.7	18.1
18:3	0.8	0.0	0.7	0.9	0.0	0.3	0.0	0.1
20:3[5]	11.3	13.7	11.9	8.0	0.0	0.7	0.0	0.0
20:4	2.2	1.7	2.3	1.7	20.7	15.1	17.4	12.5
Remainder	0.0	0.0	0.0	1.3	0.1	1.8	0.6	1.7

[1] Chickens were fed diet C-21 from zero to 7 days and supplemented diets from 7 to 50 days. These data were tested by analysis of variance using the three factor interaction as the error term. When $P < 0.05$ was taken as the necessary level of significance, it was found that addition of safflower oil to the diet increased 18:2 and 20:4 and decreased 16:1, 18:1 and 20:3; dietary methyl laurate increased 14:0; cholesterol decreased 20:4. Other effects were not significant.
[2] Dietary supplements.
[3] 5.0% Methyl laurate and 3.0% methyl myristate added to diet C-21 which contains 1.0% methyl myristate.
[4] First number is chain length, second the number of double bonds.
[5] Identification is tentative.

TABLE 4

Fatty acid analysis of tissues[1]

Safflower oil[2]	% of diet															
Laurate + myristate[3]	0 0	0 8	2 0	2 8	0 0	0 8	2 0	2 8	0 0	0 8	2 0	2 8	0 0	0 8	2 0	2 8
	Depot fat				Heart				Testis				Cerebrum			
Fatty acid[4]	% of tissue fat															
12:0	1.5	8.1	2.0	17.6	0.0	0.6	0.4	1.4	0.3	1.5	0.2	1.6	0.0	0.0	0.0	0.1
14:0	4.5	19.6	6.7	24.6	0.7	3.7	2.3	2.9	1.2	9.1	2.4	5.5	0.6	1.9	0.5	2.3
14:1[5]	0.4	0.9	0.5	2.9	0.4	0.7	1.2	0.5	1.6	1.0	1.2	1.6	2.9	2.5	2.2	2.8
16:0	24.0	19.9	25.0	15.4	16.4	14.8	10.8	13.8	20.1	18.4	27.2	25.3	25.1	25.6	24.7	33.6
16:1	9.2	3.0	8.4	5.5	4.7	6.5	1.2	0.8	5.7	5.3	3.8	5.4	3.3	1.9	1.3	3.4
17:1[5]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.9	0.5	0.8	1.7	3.4	3.1	3.6	2.5
18:0	6.2	7.4	6.0	3.8	17.3	14.6	18.0	19.8	14.4	12.8	12.6	16.2	20.3	21.9	26.8	21.0
18:1	54.5	40.8	35.9	18.4	38.6	34.8	17.5	12.8	34.2	37.0	23.5	11.9	22.9	23.0	19.0	19.4
18:2	0.0	0.0	14.1	11.9	2.8	5.7	24.0	25.2	0.1	0.0	6.3	6.9	0.0	0.0	0.6	1.1
18:3	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	1.0	0.7	0.3	0.2	0.5	0.3	0.0	0.0
20:3[5]	0.0	0.5	0.0	0.0	16.3	16.4	0.4	0.5	11.9	8.7	3.8	2.4	14.6	13.3	1.5	0.9
20:4	0.0	0.0	0.0	0.0	2.2	2.0	13.6	22.4	2.3	1.5	15.6	16.5	5.5	5.4	17.0	14.4
20:5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.2	1.5	6.0	4.7	0.0	0.0	0.0	0.0

[1] Chickens fed diet C-21 from zero to 7 days and supplemented diets from 7 to 50 days. Each number represents the average of two replicates (with and without cholesterol since cholesterol had no significant effect on any fatty acid).

[2] Dietary supplements.

[3] 5.0% Methyl laurate plus 3.0% methyl myristate added to diet C-21 which contains 1.0% methyl myristate.

[4] First number is chain length, second the number of double bonds.

[5] Identification is tentative.

methyl laurate (12:0) and methyl myristate (14:0) increased the concentration of these acids, particularly 14:0, but had no effect on 18:2 or 20:4. Addition of cholesterol decreased 20:4, confirming the observation of Dam et al. ('56), and Dam and Nielsen ('57). Cholesterol had no significant effect on fatty acid composition of depot fat, heart, testis or cerebrum and therefore the data from groups with and without cholesterol were averaged in table 4. These results show that there is a much greater incorporation of dietary 12:0 and 14:0 in depot fat than in the other tissues studied.

Addition of safflower oil increased 18:2 in all tissues and increased 20:4 in all tissues studied except the depot fat. Although the composition of the tissues was markedly affected by diet, each tissue had different fatty acid patterns with the low-fat diets, and reflected the fatty acid composition of the dietary fat to different degrees. For example, the testis was the only tissue with appreciable amounts of an unidentified fatty acid with a retention time consistent with a 20:5 structure (eicosapentaenoic acid). This is in agreement with a recent study with the rat.[3] Both testis and cerebrum contain significant amounts of a fatty acid which had a retention time comparable to a heptadecenoic acid (17:1). Heart and liver contained some 18:2 with the low-fat diets, whereas in both testis and cerebrum this acid was not present. The cerebrum contained more 20:4 with low-fat diets than other tissues. The percentage of 14:0 increased from 1.8 to 7.3% in the testis when methyl myristate was added to the basal diet, whereas in the heart the increase was only from 1.5 to 3.3%.

The effects of cholesterol and safflower oil on growth were confirmed in the second study (table 5). In addition, the feeding of pure 18:2 established that the effect of safflower oil was a consequence of its 18:2 content.

In the third study (table 6) it was found that addition of 1.50% 18:2 (as methyl linoleate) resulted in a significant improvement in growth when measured over a 7-day (days 6 to 13) or 13-day (days 6 to 20) period, whereas 0.15% 18:2 had no effect. This would indicate that the requirement for 18:2 is more than 0.15% of the diet. When 1.50% 18:3 (as methyl linolenate) was added there was no significant effect on growth during the 6- to 13-day period.

In contrast with our studies, Bieri et al. ('56) reported that chickens fed fat-free diets grow as well as chickens fed corn oil-supplemented diets for 6 to 8 weeks. In order to determine whether the difference in diets used was influencing the rapidity of response to unsaturated fatty acids, the diet used by Bieri and co-workers was fed in the third experiment (table

[3] Kirschman, J. D., and J. G. Coniglio 1960 Polyunsaturated fatty acids in normal growing rats. Federation Proc., *19:* 221 (abstract).

TABLE 5

Effect of unsaturated fatty acids and cholesterol on growth

Supplements to diet C-21-A			Gain[1]	Feed conversion
Cholesterol	Safflower oil[2]	Methyl linoleate[3]		
%	%	%	*gm*	*gm feed/ gm gain*
0	0	0	198	1.92
2	0	0	155	2.09
0	2	0	257	1.77
0	5	0	259	1.81
2	2	0	253	1.73
2	5	0	274	1.70
2	0	1.55	238	1.75

[1] From 7 to 28 days, the least significant ($P < 0.05$) difference between means is 26 gm.
[2] Added isocalorically by replacing 3.8% glucose with cellulose.
[3] Prepared by the Hormel Institute, Austin, Minnesota; this contained 96.9% linoleate, 2.5% oleate, 0.60% linolenate.

TABLE 6

Effect of methyl linoleate and methyl linolenate on early growth response in the chicken

Supplement	Average gain[1]	
	6–13 days[2]	6–20 days[3]
	gm	gm
None (diet C-21)	62	168
0.15% Methyl linoleate	63	172
1.50% Methyl linoleate[4]	69	192
1.50% Methyl linolenate[4]	62	—[5]
0.5% L-Arginine·HCl (Arg)	66	173
2.0% Safflower oil	71	194
0.5% Arg+2.0% safflower oil	73	205
None (diet C-20-G-8)[6]	60	165
Same+4.0% safflower oil	72	205

[1] Triplicate groups of 12 birds each. All birds were fed the appropriate basal diet for 6 days. They weighed 81 gm at this time.
[2] Differences over 6 gm are significant ($P < 0.05$).
[3] Differences over 19 gm are significant ($P < 0.05$).
[4] Obtained from the Hormel Institute, Austin, Minnesota. The methyl linoleate contained 96.9% linoleate; the methyl linolenate contained 99.3% linolenate.
[5] This treatment discontinued at 13 days.
[6] Bieri et al. ('56). This diet contains 20% casein and 8% gelatin.

6). Since their diet was higher in arginine, this amino acid was also studied as a variable. The results of this experiment showed that the rapidity and extent of the response to unsaturated fatty acids was comparable for all of the diets.

DISCUSSION

Cholesterol

Cholesterol feeding caused a decrease in 20:4 fatty acid concentration in liver but in none of the other tissues analyzed. It is possible that the growth of the chicken is extremely sensitive to variations in 20:4 level in specific tissues, such as the liver. An alternative explanation may be based on a shift in the location of 20:4 within the cell. When cholesterol is fed to rats, the cholesterol ester portion of the lipid in the liver increases (Ridout et al., '52; Patil and Magar, '60), whereas the phospholipid portion decreases (Patil and Magar, '60). Moreover cholesterol is preferentially esterified with unsaturated fatty acids (Swell and Treadwell, '55; Riley and Nunn, '60). It is possible therefore that the phospholipid concentration and the EFA content in the phospholipid is reduced with cholesterol feeding. If it is assumed that the primary need for EFA is as a component of phospholipids, then the net effect of cholesterol feeding would be a reduction in the EFA content at critical sites of EFA function (probably mitochondria) with a resultant reduction in growth.

Saturated fatty acids

Based on these studies it is unlikely that the growth-depressing effect of saturated fatty acids can be explained on the basis of the inhibition of 18:2 or 20:4 deposition or synthesis. Although the percentage of tissue SFA increased when SFA were added to the diet there was no decrease in EFA percentage in tissues, rather there was decrease in the monoenoic acids. Addition of saturated fatty acids may result in a shift in EFA concentration within the cell from active esters (as phospholipid) to triglycerides. Alternatively, the increase in 12:0 and 14:0 concentration in tissues may be affecting the structure of critical lipids sufficiently to alter the same metabolic function influenced by EFA. Whether feeding of 16:0 or 18:0 would produce similar effects needs further study.

Linoleate, linolenate, and arachidonate as essential fatty acids

The results of the present studies show that 18:2 but not 18:3 will stimulate growth of chickens fed diets free of unsaturated fatty acids. This is in agreement with studies with the rat (Hume

et al., '40; Greenberg et al., '50; Thomasson, '53) demonstrating little EFA activity from the usual isomer of linolenate and extends the concept (Olsen, '59) that only those unsaturated fatty acids containing the configuration CH_3–$(CH_2)_4$ $(CH = CH$ –$CH_2)_n$–R can function as EFA.

Arachidonate (20:4) has been reported to be more potent than linoleic in curing EFA deficiency in rats (Turpeinen, '38; Smedley-McLean and Nunn, '40; Greenberg et al., '51). In our studies (table 4) 18:2 could not be detected in cerebrum and testis from chickens fed EFA-free diets for 50 days, whereas appreciable amounts of 20:4 could still be measured. Histological examination indicated that these tissues were normal. The apparent normal functioning of a tissue containing no detectable 18:2 suggests that there is no specific metabolic requirement for 18:2 and that the metabolic requirement for EFA may be limited to a requirement for $CH_3(CH_2)_4$ $(CH = CH–CH_2)_n$–R (a structure found in 20:4, 22:4 and 22:5).

Early growth response

These studies indicated that the young chicken has an acute requirement for EFA. The response to dietary 18:2 as early as two weeks of age suggests that a rapid depletion of EFA from the tissues occurs. In addition growth apparently can be affected if the percentage 18:2 or 20:4 is only slightly lowered in critical lipid structures involved in metabolic reactions or in transport. The discrepancy between the present studies and that of Bieri et al. ('56) cannot be explained on the basis of diet. In our experiments chickens fed Bieri's diet grew at a rate of 12 gm per day rather than 9 gm per day as reported by Bieri. This more rapid growth rate could have been a reflection of either a different genetic potential or a different environment or both. Our results suggest that the EFA requirement may be related to rapid growth rate. The depletion of 18:2 and 20:4 in the liver and heart of chickens used in the present studies was also more rapid than that reported by Bieri et al. ('57). Depletion rate may be controlled by genetics and may also contribute to the difference between studies.

SUMMARY

Addition of safflower oil or methyl linoleate, but not methyl linolenate, to purified diets free of unsaturated fatty acids resulted in an immediate (within 7 days) growth response in chickens. Addition of a mixture of methyl myristate and methyl laurate depressed growth, and addition of cholesterol to these saturated fatty acids depressed growth further. These growth-depressing effects were largely overcome by addition of a source of linoleic acid.

Analysis of depot fat, heart, liver, testis and cerebrum using gas-liquid chromatography revealed that dietary linoleic acid profoundly affected the fatty acid composition of tissues analyzed. In tissues of chickens fed linoleic acid-free diets the level of fatty acid tentatively identified as a C-20 triene was elevated and the linoleic and arachidonic acid content was very low; there was no detectable ($< 0.1\%$) linoleic acid in testis, cerebrum, or depot fat, whereas all tissues except depot fat contained at least 1.5% of arachidonic acid. Methyl laurate and methyl myristate supplementation increased tissue levels of these fatty acids but had no effect on the per cent linoleic acid or arachidonic acid. Addition of linoleic acid resulted in a decrease in percentage of C-20 triene and an increase in the linoleic and arachidonic acid in all tissues analyzed.

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Chick Liver-Storage Bioassay of Alpha-Tocopherol: METHODS[1,2,3]

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Various bioassays have shown the relative biopotency of *d*, versus *dl*,α-tocopheryl acetate to be 1.34 using the precise chick liver-storage bioassay (Pudelkiewicz et al., '60), 1.36 using fetal resorption in the rat (Harris and Ludwig, '49a), and 1.47 using percentage of hemolysis in the rat (Friedman et al., '58). The similar relative values for *d*, and *dl*,α-tocopherol are 1.31 using chick liver-storage (Pudelkiewicz et al., '60), 1.22 using cure of muscular dystrophy in the rabbit (Hove and Harris, '47), and 1.3 using percentage of hemolysis in the rat (Rose and György, '52; Friedman et al., '58). *d*,α-Tocopheryl succinate was 1.14 times more active than *dl*,α-tocopheryl acetate for the rat (Friedman et al., '58). The potency of this ester, however, which is available in an easily weighed powder form, needs to be determined for the chick.

The free α-tocopherol was as biologically active as the ester forms of the respective natural and synthetic preparations for the chick by liver-storage (Pudelkiewicz et al., '60), and for the rat by fetal resorption (Gottlieb et al., '43) and by percentage of hemolysis (Friedman et al., '58). However, *dl*,α-tocopherol was about 1.35 times more active than the *dl*,α-tocopheryl acetate for adult human beings, using blood-levels of tocopherol (Week et al., '52), and for rats fed high-fat diets in a fetal resorption bioassay (Gottlieb et al., '43).

Although the chick liver-storage bioassay is precise and gives results that are in good agreement with other bioassay methods using other animals, this, together with the analytical procedures which use molecular distillation, is time consuming. Therefore, it seemed desirable to investigate shortening the bioassay by reducing the repletion period of two weeks to one similar to that developed by Ames and Harris ('56) for bioassay of vitamin A; also to study a chromatography method, such as that using secondary magnesium phosphate (Bro-Rasmussen and Hjarde, '57a), with respect to its precision and ability to remove interfering substances from liver extracts.

EXPERIMENTAL

Day-old, White Plymouth Rock, male chicks were reared in electrically heated, wire batteries and fed the low-fat, low-tocopherol diet (Singsen et al., '54) for 14 days to deplete the chicks of their tocopherol stores. Then the smallest and largest chicks were discarded and the remaining 60% were distributed randomly by weight into groups of 8 chicks each, wingbanded, and weighed by groups. The dietary treatments were randomly assigned to the pens of chicks.

Equimolar amounts of the pure *d*, and *dl*, α-tocopheryl acetate[5] samples and the

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[3] Presented in part at the Informal Poultry Nutrition Conference in conjunction with the Federation Meetings at Chicago, Illinois, 1960, at the 49th annual meeting of the Poultry Science Association at Davis, California, 1960, and at the annual meeting of the Animal Nutrition Research Council in Washington, D. C., 1960.

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[5] The authors are indebted to Distillation Products Industries, Rochester, New York, for the natural tocopherols, and to Hoffmann-La Roche, Inc., Nutley, New Jersey, for the synthetic tocopherols and for processing the various tocopherols into sugar-coated gelatin beadlets.

d, α-tocopheryl acid succinate[5] powders were weighed into flasks, dissolved in 50 ml of warm 95% ethanol, poured onto the respective weighed amounts of basal diet and the flasks rinsed out onto these feeds three times with a total of 50 ml of 95% ethanol. The fine gelatin-sugar beadlets[6] were weighed, according to the concentration of tocopheryl acetate found by ceric sulfate titration, into beakers, mixed with part of the weighed amount of basal diet, and then poured onto the rest of the basal diet. The basal diets and the basal diets containing beadlets were also mixed with 100 ml of 95% ethanol each. The diets were mixed 10 minutes in a Hobart mixer.

In the long chick-liver storage bioassay, each group within an experiment received the basal diet supplemented with the molar-equivalent of zero, 8, or 16 mg of α-tocopheryl acetate per pound of diet. The groups of chicks were fed on an equalized basis for 14 days, all lots within the experiment being limited to the amount of feed consumed by that lot eating the least.

In order to develop the short chick liver-storage bioassay, a dose-response experiment was undertaken. The groups of chicks were fed equal amounts of diets supplemented with zero, 40, 80, 160, or 320 mg of tocopheryl acetate[7] per pound of diet for three days and then were fed equal amounts of basal diet during a three-day stabilization period. As the levels selected for this dose-response study were satisfactory, an experiment was undertaken to determine the time for maximal liver storage after feeding α-tocopherol. The chicks were fed equal amounts of a diet supplemented with 80 mg of tocopheryl acetate per pound for three days. Then the respective experimental groups were fed equal amounts of the basal diet for one, two, three, 4, 5, 6, and 8 days. Because maximum concentration of tocopherol in the liver occurred one day after the supplement was withdrawn from the diet, as would be expected from the work of Voth et al. ('58), the dose-response study was repeated. This time the chicks were fed equal amounts of the basal diet supplemented with zero, 40, 80, or 160 mg of tocopheryl acetate per pound of feed for three days and then the basal diet for one day.

As the concentration of tocopherol in the chick livers was linearly related to the milligram dosage of tocopheryl acetate in the diet, short, chick liver-storage bioassay experiments were undertaken. Each group within an experiment received the basal diet supplemented with the molar-equivalent of zero, 40, or 80 mg of α-tocopheryl acetate per pound of diet. The groups of chicks were fed equal amounts of these diets for three days and then the basal diet for another day. Chicks consumed 20 gm of supplemented ration per bird the first day and up to 25 gm of basal ration the 4th day.

At the end of the experimental periods, the groups of chicks were weighed and the chicks decapitated to exsanguinate the livers. The livers from each group were pooled, refrigerated, weighed, and homogenized in a Dumore blender. Approximately 8-gm samples of homogenate were weighed into extraction thimbles and stored in separate rubber-stoppered, plastic tubes at −20°.

METHODS

All pooled livers were analyzed in duplicate by the combination of extraction, molecular distillation, chromatography through Florex XXS[8] and colorimetric measurement of tocopherol with ferric chloride-dipyridyl as first used in this laboratory by Bunnell,[9] and described in detail by Pudelkiewicz et al. ('60).

All tocopherol and tocopheryl acetate gelatin-sugar beadlets were analyzed for tocopherol by the combination of extraction, sulfuric acid hydrolysis of only the acetate esters, and ceric sulfate titration (Lehman, '57).

The pooled livers from each of the groups in 4 experiments were analyzed in

[5] See footnote 5.

[7] Vitamin E concentrate was Myvamix, which is a proprietary product manufactured by Distillation Products, Inc., Rochester, New York, guaranteed to contain 20,000 I.U. of natural vitamin E per pound.

[8] The authors are indebted to the Floridin Company, Tallahassee, Florida, for the Florex XXS.

[9] Bunnell, R. H. 1955 The effect of fish oil and diphenyl-p-phenylene-diamine on the vitamin E metabolism of the chick. Ph.D. thesis, University of Connecticut, Storrs.

duplicate by the combination of extraction (Pudelkiewicz et al., '60), saponification (Pudelkiewicz et al., '60), chromatography through secondary magnesium phosphate (Bro-Rasmussen and Hjarde, '57a), and colorimetric measurement with ferric chloride-dipyridyl (Pudelkiewicz et al., '60). As this method of liver analysis for tocopherol consists of procedures taken from various publications, the complete method is presented here.

Reagents

Skellysolve B and *absolute ethanol* were purified and *potassium hydroxide, 2,2′ bipyridine,* and *ferric chloride* solutions were prepared as described by Pudelkiewicz et al. ('60).

Petroleum ether. Bottled, reagent grade benzin boiling over a range of 30 to 60°C was used. If the reagent grade was purchased in cans, it had to be purified. The method for purification of Skellysolve B (Pudelkiewicz et al., '60) proved satisfactory.

Ethyl ether. The anhydrous, analyzed reagent[10] was rendered peroxide-free by allowing to stand over a half-cup of Norit A and up to 100 gm of potassium hydroxide pellets in a 2-liter distillation flask and distilling before use.

Secondary magnesium phosphate. The method of Bro-Rasmussen and Hjarde ('57a) was used. A 580-gm sample of chemically pure,[11] secondary magnesium phosphate trihydrate was dried at 180°C for 24 hours and cooled over concentrated sulfuric acid in a desiccator. The 400 gm of remaining anhydrous salt was slowly added to an already boiling solution of 32 gm of anhydrous secondary sodium phosphate in 3200 ml of distilled water. The mixture was boiled for 15 minutes, cooled somewhat, filtered on a Buchner funnel through Whatman no. 2 paper, and washed three times with about 700 ml of distilled water each time. The hydrated salt was dried for at least 48 hours (easier to grind if 72 hours) at about 180°C. The dried salt was then cooled in a desiccator over concentrated sulfuric acid, ground in a ball mill for about 15 minutes, sieved to 120 mesh with the aid of the balls from the ball mill and mechanical shaking, and stored in a tightly capped, brown bottle at room temperature.

The charged secondary magnesium phosphate was checked for activity by the method described by Bro-Rasmussen and Hjarde ('57a) which involves: chromatographing a 100- to 400-μg sample of pure α-tocopherol; eluting with 100 ml of petroleum ether, then with 100 ml of a mixture of 0.5% ethyl ether in petroleum ether; and finally with 250 ml (instead of 125 ml, Bro-Rasmussen and Hjarde, '57a) of a mixture of 2% ethyl ether in petroleum ether. No charges were used that passed tocopherol by elution with 0.5% (or less) of a mixture of ethyl ether in petroleum ether or that did not show complete elution of the pure tocopherol by 250 ml of 2% ethyl ether in petroleum ether.

The activity of some charges checked by this method, although satisfactory by this criteria, failed to adsorb all the interfering reducing substances in the extract of a liver sample which was being purified. Because of this difficulty, the activated secondary magnesium phosphate was also checked by chromatographing an aliquot of the eluate from a column on paper. The column of adsorbent was washed with 50 ml of petroleum ether and then the α-tocopherol of the liver extract was eluted with 250 ml of a mixture of 2% ethyl ether in petroleum ether. This eluate was evaporated to dryness under nitrogen and the residue dissolved in 0.5 ml of ethanol. More than 0.1 ml of this was spotted on paraffin-impregnated chromatography paper and the chromatograms developed with 75% ethanol (Analytical Methods Committee, '59). The papers were sprayed with a mixture of equal amounts of 0.5% α,α-dipyridyl and 0.2% ferric chloride solutions in ethanol. The presence of more than the one α-tocopherol spot expected indicated that the adsorbent was not sufficiently active. Recoveries of pure tocopherol added to the impure samples and the stability of the colorimetric readings with ferric chloride-dipyridyl are also indicative

[10] Baker Chemical Company, Phillipsburg, New Jersey.

[11] Amend Drug and Chemical Company, New York 10.

of the completeness of the removal of interfering substances by the adsorbent. Hence, these checks can be used to indicate charges that should be discarded and to determine the amounts of extracts that can be purified by the adsorbent.

Extraction and saponification

Liver samples were extracted in Soxhlet-type apparatus for 20 hours with 75 ml of absolute ethanol, aldehyde-free (Pudelkiewicz et al., '60). After 20 hours of extraction, each flask in turn was lowered from its condenser, 2 ml of the concentrated potassium hydroxide solution was quickly added, the flask and condenser were immediately connected, and boiling was continued for another half-hour. Immediately after saponification, 70 to 75 ml of distilled water containing 1 ml of concentrated sulfuric acid were added to neutralize the excess alkali. The mixture was cooled and extracted with exactly 40 ml of purified Skellysolve B as described by Pudelkiewicz et al. ('60).

Chromatography

The method used was essentially that described in detail by Bro-Rasmussen and Hjarde ('57a). Approximately 12 gm of activated secondary magnesium phosphate per column were suspended in petroleum ether. This mixture was run into 12 by 240-mm chromatography tubes that had previously been plugged with glass wool. The chromatography column had a 6 by 120-mm delivery tube and an eluant-reservoir with capacity for more than 200 ml of fluid and with an opening for a no. 4 rubber stopper. The column was packed by about two pounds per inch^2 of pressure from a nitrogen cylinder connected to the opening of each column. When more than 200 mm of the effective length of the chromatography tube was packed with adsorbent, the adsorbent was covered with about 5 mm of anhydrous sodium sulfate.

An aliquot (10 ml) of the saponified, Skellysolve B liver-extract was pipetted into a 50-ml Erlenmeyer flask and evaporated to dryness on a steam bath under nitrogen. The residue was dissolved in 5 ml of petroleum ether and poured onto the column. The eluant was forced through by two to 8 pounds per inch^2 of pressure from a nitrogen tank to give a flow rate of two to 4 ml per minute. The column of adsorbent was never allowed to go dry. After the liver extract was washed onto the column, the column was washed with 50 ml of petroleum ether, the receiver was changed, and the α-tocopherol was eluted with a total of 250 ml of a mixture of 2% ethyl ether in petroleum ether. *This 200-mm column of secondary magnesium phosphate could absorb only the non-tocopherol reducing substances in less than 15 ml of the 40 ml of Skellysolve B extract of about 8 gm of liver.* Perhaps this fact can explain why this method has given nonreproducible results in some laboratories. As little as 5 μg of tocopherol were chromatographed when the livers of chicks fed the basal diets were analyzed.

The eluate was evaporated to dryness under nitrogen on a steam bath and dissolved in 10 ml, or more, of absolute ethanol, aldehyde-free. Enough ethanol was added to insure that the solution contained less than 60 μg of tocopherol in an 8-ml aliquot. Color was developed by the addition of 1 ml of 0.5% dipyridyl solution and 1 ml of 0.2% $FeCl_3$ solution. Exactly 15 seconds after the addition of the $FeCl_3$ the solution was read in an Evelyn colorimeter at 515 mμ (Pudelkiewicz et al., '60).

Feed

Basal diets were analyzed by either of the methods used for liver. If tocopheryl esters were added to the basal diet, the supplemented feeds were saponified with 4.5 ml of the concentrated potassium hydroxide solution in the vapors of ethanol, after extraction as described above for liver analysis. Hence, after 30 minutes of saponification, the mixture was acidified with 2 ml of concentrated sulfuric acid in 70 to 75 ml of water.

The method of chromatography through secondary magnesium phosphate proved satisfactory for determining both α- and non-α-tocopherols in feed. After the α-tocopherol had been eluted with 250 ml of mixture of 2% ethyl ether in petroleum ether, the other tocopherols were eluted by 250 ml of a mixture of 7% ethyl ether in petroleum ether (Bro-Rasmussen and

Hjarde, '57b), and measured colorimetrically with ferric chloride-dipyridyl exactly as for α-tocopherol. *Here again the 200-mm column could purify only up to half the extract from 8 to 10 gm of feed.*

STATISTICAL ANALYSIS

With the long, chick liver-storage bioassay, 10 experiments were undertaken to determine the relative biopotency of *d*, versus *dl*, α-tocopheryl acetate as well as to determine the biopotency of *d*- α-tocopheryl acid succinate. Six of these experiments were replicates in time, for the same tocopherol samples (lot numbers) were used in each. For the same reason, the other 4 experiments were replicates in time. With the short, chick liver-storage bioassay, 4 experiments that were replicates in time were undertaken to determine the relative biopotencies of the *d*, and *dl*, α-tocopheryl acetates, α-tocopherols, and *d*, α-tocoperyl acid succinate. These respective series of experiments were grouped together for analysis of variance and for the multiple slope-ratio assay described by Finney ('52). The equations for this analysis were solved by the abbreviated Doolittle method described by Anderson and Bancroft ('52). This statistical approach makes it possible to determine simultaneously the slopes of the dose-response relation for each of the tocopherol samples assayed in a given series of experiments. The multiple slope-ratio assay is valid if the dose-response relations for each of the samples studied are linear and if the slopes for these dose-response relations converge, preferably at the basal level point.

The dose-response studies were statistically analyzed by standard procedures of regression and analysis of variance as outlined by Snedecor ('56).

The duplicate variance for 46 samples analyzed in duplicate by the two methods of analysis of liver for tocopherol were compared by the Pitman-Morgan test for correlated mean squares (Cochran, '45). Hence, it is possible to compare the precision of results obtained from two methods used to analyze a series of samples.

RESULTS

The average concentration of tocopherol in the livers from chicks in the different dietary groups for the 4 long bioassay experiments, and the 4 short bioassays are presented in table 1. These data show the direct relationship between the level of tocopherol fed and the level of tocopherol observed in the liver. This relationship was found to be linear. The slopes of these lines converged at a Y-intercept (table 1) which was essentially identical to the amount of tocopherol present in the livers of those chicks fed the basal diet (table 1).

The average liver weight for all the chicks used in the 4 long bioassay experiments was 9.8 gm per liver, with a range of 8.9 to 11.2. These chicks consumed on the average 414 gm of supplemented feed per chick, with a range of 393 to 423 for the 4 experiments. In the 4 short bioassay experiments, the average liver weight was 6.7 gm per liver with a range of 6.2 to 7.5. These chicks consumed on the average 66 gm of supplemented feed per chick, with a range of 63 to 69 for the 4 experiments. The basal diet contained total tocopherols equivalent to 7 to 12 mg of tocopheryl acetate (which was present in the feed as the free form) and the equivalent of three to 4 mg of α-tocopheryl acetate per pound of feed.

The mean α-tocopherol values for these respective groups of experiments with their standard errors and coefficients of variation are also presented in table 1. The mean for the series of 6 long bioassay experiments, not presented in table 1, was 9.5 μg per gm of liver with a standard error of ± 0.1 and a coefficient of variation of 9.3%. From the coefficient of variation of these three series of bioassay experiments, it can be inferred that these bioassay methods are relatively precise and that the long and the short bioassay can be executed with essentially the same degree of precision.

The slopes for the respective tocopherol samples studied, with their standard errors, and the relative biopotencies for the forms of tocopherol studied in both the long and short bioassay are summarized in table 2. For each sample studied in each of these series of bioassay experi-

TABLE 1

Comparison of two methods of bioassay of α-tocopherol (average liver tocopherol)

Tocopherol sample	Long bioassay[1]			Short bioassay[2]		
	Molarequivalents, mg α-tocopheryl acetate/pound of feed					
	0	8	16	0	40	80
	Liver tocopherol treatment means[3] (μg/gm)					
dl,α-Tocopheryl acetate[4]						
Pure 1	2.4	7.5	12.6	2.9	—	—
Pure 2	2.2	7.5	11.8	2.4	—	—
Beadlet 3	—	8.3	12.6	—	14.8	24.6
Pure 4	—	—	—	—	14.1	27.8
Beadlet 4	—	—	—	—	14.6	28.6
Beadlet 5	—	—	—	—	13.6	25.7
dl,α-Tocopherol-beadlet	—	—	—	—	14.3	25.5
d,α-Tocopheryl acetate[4]						
Pure 1	—	7.9	13.6	—	—	—
Beadlet 2	—	8.1	15.4	—	—	—
Pure 3	—	—	—	—	16.1	32.1
d,α-Tocopherol-beadlet	—	—	—	—	18.0	32.4
d,α-Tocopheryl acid succinate						
Powder 1	—	5.9	9.8	—	10.8	16.9
Y-intercept	2.4	—	—	2.6	—	—
Mean of the experiments	—	8.9	—	—	18.3	—
Standard error of the mean	—	±0.1	—	—	± 0.2	—
Coefficient of variation	—	12.4%	—	—	10.3%	—

[1] Long bioassay involved two weeks of tocopherol supplementation.

[2] Short bioassay involved three days of tocopherol supplementation followed by one day of feeding basal diet.

[3] Four experiments that were replicates in time for each of the two bioassay procedures were statistically analyzed together.

[4] These numbers represent different lot numbers of the respective forms of tocopherol fed in the bioassay experiments.

ments, the relative values were determined by dividing the slope of the dose-response relation for each of the respective samples studied by the average of the slopes of the dose-response relation obtained for *dl*, α-tocopheryl acetate. Hence, these data presented in table 2 show that the *d*, α-tocopheryl acid succinate is poorly utilized by the chick, whereas the *d*, α-tocopheryl acetate was the most available form, as expected from the work of Pudelkiewicz et al. ('60).

In the dose-response study undertaken to shorten the bioassay, the chicks were fed zero, 40, 80, 160, or 320 mg of tocopheryl acetate per pound of diet for three days and then the basal diet for three days. The equation for the relation of micrograms of tocopherol per gram of liver to the milligrams of tocopheryl acetate per gram of feed was: $y = 1.9 + 0.155$ X, with ± 1.0 and + 0.006 as the standard error of the y-intercept and of the slope, respectively. In the dose-response studies in which the chicks were fed zero, 40, 80, or 160 mg of tocopheryl acetate per pound of diet for three days and then the basal diet for one day, the equation for the relationship of micrograms of tocopherol per gram of liver to the milligrams of tocopheryl acetate per gram of feed was: $y = 2.9 \pm 0.8 + (0.366 \pm 0.003)$ X (liver analyzed for total tocopherol by molecular distillation followed by Florex-chromatography) and $y = 3.3 \pm 0.9 + (0.355 \pm 0.003)$ X (liver analyzed for α-tocopherol by saponification followed by chromatography through secondary magnesium phosphate). As maximum concentration of tocopherol in the liver occurs within one day after tocopherol supplementation is stopped, the liver-storage of tocopherol was much more related to intake of tocopherol (steeper slope) if livers were taken after one day of withdrawal than after three days of withdrawal of tocopherol supplementation.

Since the variability among groups fed the same level of tocopherol in the dose-response studies increased with increasing concentration of tocopherol in the liver,

TABLE 2

Comparison of two methods of bioassay of α-tocopherol (relative values)

Tocopherol sample	Long bioassay[1]				Short bioassay[2]	
	Slope		Relative value[3]		Slope	Relative value[3]
	Series of experiments[4]					
	A	B	A	B		
dl,α-Tocopheryl acetate[5]						
Pure 1	0.671	0.639	0.96	1.01	—	—
Pure 2	0.682	0.600	0.98	0.95	—	—
Beadlet 3	0.731	0.660	1.05	1.04	0.281	0.94
Pure 4	—	—	—	—	0.309	1.03
Beadlet 4	—	—	—	—	0.319	1.07
Beadlet 5	—	—	—	—	0.285	0.96
Pure 6	0.701	—	1.01	—	—	—
dl,α-Tocopherol-beadlet	—	—	—	—	0.287	0.96
d,α-Tocopheryl acetate[5]						
Pure 1	0.812	0.700	1.17	1.10	—	—
Beadlet 2	—	0.794	—	1.25	—	—
Pure 3	—	—	—	—	0.362	1.21
d,α-Tocopherol-beadlet	—	—	—	—	0.375	1.25
d,α-Tocopheryl acid succinate[5]						
Powder 1	—	0.462	—	0.73	0.183	0.61
Powder 2	0.539	—	0.77	—	—	—
Standard error of the slope	±0.025	±0.038	—	—	±0.013	—

[1] Long bioassay involved two weeks of tocopherol supplementation.

[2] Short bioassay involved three days of supplementation followed by one day of feeding basal diet. Four experiments that were replicates in time were undertaken.

[3] Slope of the response from the respective sample divided by the average slope of the response from all the *dl,α*-tocopheryl acetate samples studied in each series of experiments that were replicates in time.

[4] Series A involved 6 experiments that were replicates in time, and series B involved 4 experiments that were replicates in time.

[5] These numbers represent different lot numbers of the respective forms of tocopherol fed in the bioassay experiments.

it seemed desirable to transform the liver tocopherol concentrations for these experiments to their respective logarithms (base 10) (Bunnell, '55) for a repetition of the regression analysis. As this minimizes differences in the variability associated with the level of tocopherol intake, a more valid estimate of the error variance can be obtained. Since more than 99% of the variability between the levels in these dose-response experiments was accounted for by the linear regression of tocopherol concentration in the liver on tocopherol intake, both the tocopherol concentration in the liver and the tocopherol intake needed to be transformed to the respective logarithms. Using the equivalents of α-tocopheryl acetate found in the diet by analysis, the levels of α-tocopherol fed were 4, 41, 78, or 151 mg of α-tocopheryl acetate per pound of feed. Linear regression of the logarithms of the liver tocopherol concentration (μg per gm) on the logarithms of the α-tocopheryl acetate intake (mg per pound) for the three-day supplementation, one-day stabilization period, gave the following equations: $y = -0.10 \pm 0.02 + (0.854 \pm 0.015)$ X (liver analyzed for total tocopherol by molecular distillation plus Florex-chromatography) and $y = -0.10 \pm 0.03 + (0.852 \pm 0.016)$ X (liver analyzed for α-tocopherol by saponification plus secondary magnesium phosphate-chromatography).

These linear regression equations, the slopes of the lines for the respective tocopherol samples studied in one short, chick liver-storage bioassay experiment (table 3), and the duplicate variances for the two methods of tocopherol analysis (table 4) indicate the similarity of the results obtained by either molecular distillation plus Florex-chromatography or saponification plus secondary magnesium phosphate-chromatography. All of the first of the duplicate samples for each experi-

TABLE 3

Comparison of two methods of purification of liver extracts (relative utilization of tocopherol in the bioassay)

Tocopherol sample	Molecular distillation Florex-chromatography		Saponification $MgHPO_4$-chromatography	
	Slope	Relative value[1]	Slope	Relative value[1]
One short, chick liver-storage bioassay experiment				
dl,α-Tocopheryl acetate[2]				
Beadlet 3	0.286	0.91	0.308	0.96
Pure 4	0.318	1.01	0.330	1.02
Beadlet 4	0.367	1.17	0.366	1.14
Beadlet 5	0.283	0.90	0.282	0.88
dl,α-Tocopherol-beadlets	0.278	0.89	0.307	0.96
d,α-Tocopheryl acetate[2]				
Pure 3	0.432	1.38	0.423	1.31
d,α-Tocopherol-beadlet	0.426	1.36	0.421	1.32
d,α-Tocopheryl acid succinate[2]				
Powder 1	0.202	0.64	0.244	0.76
Standard error of the slope	±0.018		±0.018	

[1] Slope of the response from the respective sample divided by the average slope of the response from the four *dl*,alpha-tocopheryl acetate samples studied in this experiment.

[2] These numbers represent different lot numbers of the respective forms of tocopherol fed in the bioassay experiments.

TABLE 4

Comparison of two methods of purification of liver extracts (precision of these analytical methods)

	Molecular distillation Florex-chromatography		Saponification $MgHPO_4$-chromatography
	Total tocopherol		α-Tocopherol
		μg/gm liver	
Range	2.3–67.9		2.2–63.3
Mean	22.1		22.6
No. of samples	46		46
Duplicate variance[1]	2.533		2.176
Correlation coefficient		0.992	
Coefficient of variation	7.2%		6.5%

[1] Duplicate variance $= \frac{S(\text{d Dup.}^2)}{2N}$; N = number of samples, in this case 46; d Dup. = difference between duplicate analyses.

ment were analyzed by both methods before the second of the duplicate samples from this experiment were analyzed. In the case of secondary magnesium phosphate-chromatography, each of the duplicate samples was always passed through batches of this adsorbent charged at different times. Most of the secondary magnesium phosphate used was from one lot number. For all 46 samples analyzed by both methods (table 4), the duplicate variance for the method of saponification followed by secondary magnesium phosphate-chromatography was less ($P < 0.01$ by the Pitman-Morgan test for correlated mean squares, Cochran, '45) than for the method of molecular distillation followed by Florex-chromatography.

In the analysis for total tocopherol, the extract from 5 to 6 gm of liver was passed through the molecular distillation plus Florex-chromatography procedure, whereas in the analysis for α-tocopherol, the extract from only 2 gm of liver was passed through the column of secondary magnesium phosphate. Hence, the final ethanol solution after Florex-chromatography contained more than 12 μg of tocopherol, whereas only 5 μg of tocopherol were present in the final ethanol solution after

chromatography through secondary magnesium phosphate. Although 5 μg of tocopherol can be measured colorimetrically, this is close to the minimum level of tocopherol that can be detected. Hence, the colorimetric measurements are less precise when 5 μg of tocopherol are present than when 12 μg of tocopherol are present.

It was observed that when more than half of the extract of about 8 gm of liver was put on a column, the mixture of 2% ethyl ether in petroleum ether caused the elution of yellow substances and substances that reacted with ferric chloride-dipyridyl. Paper chromatography of these eluates showed a large amount of yellow or reducting substances or both, at the origin plus the expected α-tocopherol spot. When ¼th of the extract of about 8 gm of liver was put on the column, the mixture of 2% ethyl ether in petroleum ether apparently caused only the elution of α-tocopherol. At least paper chromatograms showed only the expected spot of α-tocopherol. Therefore, it was concluded that secondary magnesium phosphate has a limited adsorptive ability.

DISCUSSION

The results of both the long and the short bioassays indicated that the *d*, and *dl*, α-tocopheryl acetates were utilized to essentially the same relative extent under the two sets of conditions. These relative biopotencies are similar to those obtained previously for the chick, using the long bioassay (Pudelkiewicz et al., '60), and to those reported for the rat, using percentage of hemolysis (Friedman et al., '58) and using fetal resorption (Harris and Ludwig, '49a). Likewise, the free and acetate forms of the respective *d*, and *dl*, α-tocopherols were as well utilized by the chick in this short bioassay as by the chick in the long bioassay (Pudelkiewicz et al., '60), as by the rat in the percentage of hemolysis bioassay (Friedman et al., '58), and as by the rat fed a low-fat diet in a fetal resorption bioassay (Gottlieb et al., '43).

Both the long and the short bioassays show that the α-tocopheryl acid succinate is poorly utilized by the chick. As the rat bioassays by fetal resorption (Harris and Ludwig, '49b) and by percentage of hemolysis (Friedman et al., '58) showed the natural succinate to be equal to or slightly less well utilized than the natural acetate, it would appear that the chick is much more limited than the rat in its ability to hydrolyze the succinate ester. Perhaps the esterase(s) in the chick's gut is more active with monocarboxylic acid esters, or perhaps the free carboxyl group of the tocopheryl acid succinate reacts with other substances in the gut to form an insoluble complex, with the result that the succinate is poorly utilized. The succinate form appeared to be relatively less well utilized by the chick, using the short bioassay than using the long bioassay, but further work is needed to confirm this.

The method of saponification and chromatography through secondary magnesium phosphate, developed by Bro-Rasmussen and Hjarde ('57a) for analysis of oils, foods, and feedstuffs for α-tocopherol, has been shown in this study to be a precise substitute for the method of molecular distillation plus Florex-chromatography, which Bunnell ('55) showed to be superior to Florex-chromatography for purifying liver extracts for the determination of total tocopherol. Secondary magnesium phosphate, however, was limited in its ability to adsorb substances in liver extracts. Hence, smaller amounts of liver extracts must be purified and livers containing very low levels of tocopherol are analyzed with less precision by this method than by the older method.

SUMMARY

The results of both a long (two-week supplementation) multiple slope-ratio chick liver-storage bioassay and a short (three-day supplementation, one-day stabilization) bioassay to tocopherol indicated that the different forms of α-tocopherol were utilized to the same relative degree under the two sets of conditions. The *d*,α-tocopheryl acid succinate in relation to the *dl*,α-tocopheryl acetate was only 0.61 (short bioassay) to 0.73 and 0.77 (long bioassay) as well utilized by the chick.

The method of Bro-Rasmussen and Hjarde ('57a), when modified as to the saponification procedure, with the same

procedure of chromatography through secondary magnesium phosphate, and with a modified method of reading with ferric chloride-dipyridyl, was shown to be a possible substitute for the method of molecular distillation and Florex-chromatography for analysis of liver for α-tocopherol. Limitations of the method of saponification followed by secondary magnesium phosphate-chromatography were reported.

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Effect of Various Vitamin Deficiencies on Citric Acid Metabolism in the Rat[1]

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The metabolism of citric acid in higher animals is now known to be influenced by vitamin D (Dickens, '41; Nicolaysen and Nordbø, '43; Harrison and Harrison, '52; Freeman and Chang, '50; Waasjö and Eeg-Larsen, '51; Bellin and Steenbock, '52; Steenbock and Bellin, '53; Carlsson and Hollunger, '54) and by parathyroid hormone (Dickens, '41; Freeman and Chang, '50; Alwall, '44; Neuman et al., '56; Firschein et al., '58). These relationships prompted further investigation of the possible role played by vitamins other than vitamin D in regulating tissue and urinary citrate levels. Such information might shed considerable light on the mechanism of citrate accumulation after vitamin D administration and be of value in studying the relationship between the effect of vitamin D on citrate and on Ca metabolism. In this respect, it has been reported that cortisol prevents the action of vitamin D on bone and serum citrate without affecting the action of vitamin D on Ca and P metabolism (Harrison et al., '57, '58).

The possibility that certain vitamin deficiencies might greatly affect citrate metabolism seemed quite likely from their known metabolic functions. For example, thiamine and pantothenic acid could be expected to alter citrate levels because of their coenzymic role in pyruvate and acetate metabolism, respectively. Sober et al. ('40) reported that thiamine deficiency resulted in a diminished urinary citrate. Smith and Meyer ('41), however, presented evidence that this was due to inanition.

To our knowledge, no further systematic investigations of the influence of vitamin deficiencies on citrate metabolism have been reported. In the present paper are presented the results of a study of the effect of deficiencies of pantothenic acid, thiamine, biotin, vitamin A and vitamin B_6 on urinary and tissue citrate.

METHODS

Young, male weanling rats of the Sprague-Dawley strain were used in all experiments. The diet fed was essentially that used by Steenbock and Herting ('55) and consisted of the following: (in per cent) protein, 18; cottonseed oil,[2] 10; roughage,[3] 3; Ca- and P-free salts, 2; vitamins; $CaCO_3$, 1.09; equimolar mixture of KH_2PO_4 and K_2HPO_4 to give 0.3% of P and glucose monohydrate[4] to 100%. The protein source was generally vitamin-test casein[5] plus 0.2% of L-cystine, except in the case of biotin deficiency when it was raw egg white, and in thiamine deficiency when we used steamed egg white. The control diet for the biotin experiment also contained steamed egg white as the protein source. The salt mixture has been described previously (Bellin and Steenbock, '52). The vitamin addition supplied the following milligram quantities per kilogram of diet: thiamine, 5; riboflavin, 5; pyridoxine, 5; Ca pantothenate, 28; nicotinic acid, 20; biotin, 0.1; inositol, 100; folic acid, 0.2; choline, 500; and vitamin B_{12}, 0.02. In addition, the rats received every three days a supplement of fat-solu-

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[2] Wesson Oil, The Wesson Oil Company, New Orleans, Louisiana.

[3] Cellu Flour, Chicago Dietetic Supply House, Chicago.

[4] Cerelose, Corn Products Refining Company, New York.

[5] "Vitamin-Free" Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

ble vitamins in cottonseed oil* which supplied 70 μg of β-carotene, 105 μg of 2-methyl, 1,4-naphthoquinone and 875 μg of α-tocopherol weekly. Where indicated each rat received 75 IU of vitamin D_3 every three days as a solution of crystalline calciferol in cottonseed oil.

In each experiment the rats were divided equally into 4 groups. Group 1 received the basal diet (deficient both in vitamin D and the vitamin being tested); group 2, basal diet plus the vitamin being tested; group 3, basal diet plus vitamin D; and group 4 (control group), basal diet, plus vitamin D and the test vitamin. The animals were housed in individual overhanging screen-bottom cages for 6 days and were then transferred to circular metabolism cages for the collection of urine. Rats had access to unlimited quantities of food for 4 one-hour periods daily in the experiments dealing with thiamine, pantothenic acid and biotin deficiences. In all other experiments they had access to food at all times. At the end of each experiment, the rats were killed by a sharp blow on the head followed by decapitation. Blood was collected and the serum used for citrate analysis following deproteinization with trichloroacetic acid. Tissues were quickly removed and quick-frozen in liquid air. They were ground and extracted in a mortar containing cold 10% trichloroacetic acid and ignited sand. The residue was re-extracted and the combined filtrates were analyzed for citrate. Femurs were removed, cleaned of adhering tissue, extracted with diethyl ether for 24 hours, ground to a powder and extracted 4 times with 1 N sulfuric acid. The combined sulfuric acid extract was analyzed for citric acid content. All citric acid analyses were carried out according to the method of Speck et al. ('46).

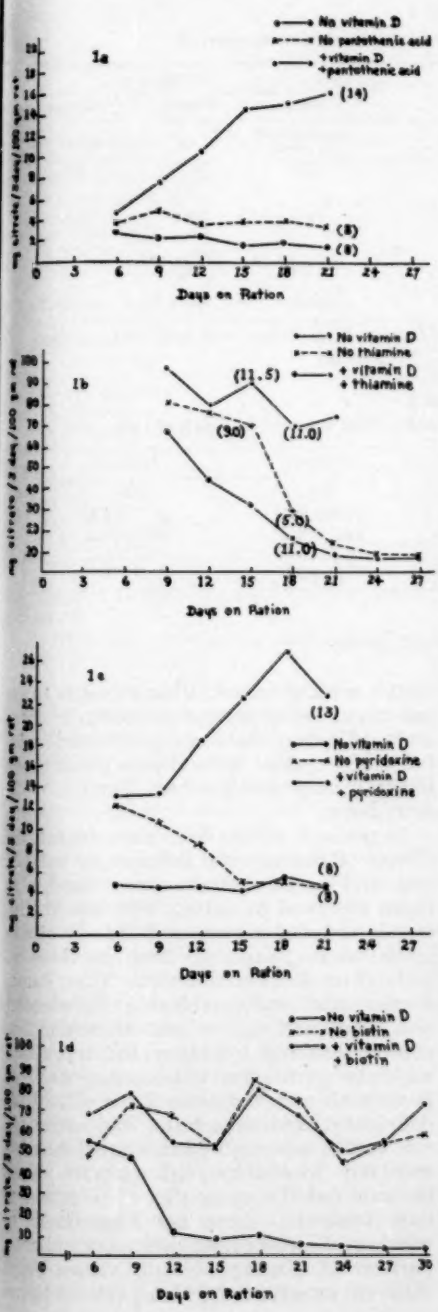
Growth and food consumption data were obtained in all experiments. To conserve space, these are reported only for experiments in which they were of critical importance. The growth data in all cases were in agreement with previous observations (Steenbock and Herting, '55). The expected vitamin deficiency symptoms were noted in each case, namely, reduced growth (all vitamins), loss of hair (biotin), dermatitis (vitamin B_6), xerophthalmia (vitamin A) and anorexia (vitamin B_1) (Robinson, '51). In each instance the deficiency symptoms could be reversed by the specific vitamin in question.

In the experiments in which oxidation studies were carried out, kidneys were quickly removed from the animals and chilled in ice-cold isotonic KCl. Ten per cent homogenates were prepared at 0°C in isotonic KCl with a Potter-Elvehjem homogenizer fitted with a Teflon pestle. Oxygen consumption was measured by standard Warburg techniques at 37°C with air as the gas phase. The reaction mixture contained 0.2 ml of homogenate, 40 μmoles of PO_4 buffer, pH 7.3, 6 μmoles of ATP, 15 μmoles of pyruvate, 10 μmoles of oxalacetate, 0.08 μmoles of cytochrome C, 10 μmoles of $MgCl_2$, and isotonic KCl to 3 ml. The center well contained 0.2 ml of 10% KOH absorbed on a 2-cm² filter paper. The oxidations were continued for 30 minutes and the results are expressed as microliters of oxygen consumed per hour per milligram of dry tissue (Q_{O_2}). Dry tissue weights were arbitrarily taken as 20% of the fresh tissue weights.

RESULTS

Pantothenic acid and thiamine deficiencies were expected to affect citrate excretion because of their known metabolic functions. In agreement with this, a lack of pantothenic acid was observed to depress urinary citrate excretion almost to the same degree as a lack of vitamin D (fig. 1a); but although the feeding of a thiamine-deficient ration depressed urinary citrate in agreement with the observations of others (Sober et al., '40; Smith and Meyer, '41), this occurred only after a severe decrease in food consumption (fig. 1b). When the food intake was maintained at the level of intake of deficient rats and thiamine was given, no increase in citrate excretion was noted (table 1). Furthermore, paired feeding of thiamine-fed rats with the thiamine-deficient rats resulted in comparable urinary citrate values (table 1). When excretion and tissue levels of citrate in the thiamine-deficient animals were examined after a 15-day depletion period and when food con-

* See footnote 2.



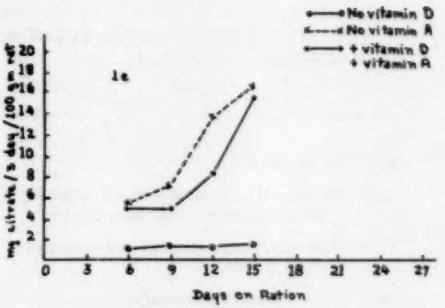


Fig. 1 Urinary citrate during vitamin deficiency; (1a) pantothenic acid, (1b) thiamine, (1c) pyridoxine, (1d) biotin, and (1e) vitamin A. The values in parentheses represent food consumption in grams per day for the last day of the experiment except where otherwise indicated. There were usually 6 and at least 4 rats in each group.

sumption had not yet decreased appreciably, no effect of thiamine deficiency was noted (fig. 1b, tables 1 and 3). Evidence that at this stage a deficiency of the vitamin prevailed is provided by the data on the oxidation of pyruvate by kidney homogenates (table 2). They show that in the deficient animals pyruvate oxidation was drastically reduced and that further depletion (namely, after receiving the diet for 21 days instead of 15 days) resulted in no further diminution of oxidative activity. These results showing that a lack of thiamine has no direct effect on tissue and urinary citrate levels agree with the conclusions of Smith and Meyer ('41). In deficiencies other than thiamine (except that of vitamin A, in which instance no metabolic effect of a deficiency was obtained) no complication due to food consumption was encountered. All these animals consumed food in proportion to body weight (fig. 1).

Of the remaining deficiencies studied, only vitamin B_6 deficiency reduced urinary citrate markedly (fig. 1c). Metabolically this reduction is difficult to rationalize on the basis of what is known. It is possible that with a decrease in the activity of the transaminases that certain key amino acids feed into the tricarboxylic acid cycle at a reduced rate as for example at the α-ketoglutarate, pyruvate and oxalacetate stages. Another possibility is that vitamin

TABLE 1

Effect of vitamin B_1 and food restriction on citrate content[1]

Group	Citrate		
	Urine	Kidney	Serum
	mg/day/100 gm of rat	μg	mg/100 ml
(1) No vitamin B_1	2.5	27.0	3.0
(2) Vitamin B_1, 1 mg/day on days 18–24, food restricted to that of days 15–18	2.9	26.0	2.5
(3) Plus vitamin B_1 throughout, pair-fed with group 1	1.4	16.3	2.4
(4) Plus vitamin B_1 throughout	15.3	36.1	4.0

[1] Four rats/group; fed their respective diets for 24 days. Urine was collected on days 21 to 24.

TABLE 2

Pyruvate oxidation[1] *by kidney homogenates from vitamin B_1-deficient rats*

	15 Days[2]	21 Days[2]
	Q_{O_2}	Q_{O_2}
No vitamin B_1, no vitamin D	27.8±4.1[3]	26.7±4.8[3]
No vitamin B_1	26.1±6.2	23.7±5.1
No vitamin D	69.0±3.0	79.3±4.6
Plus vitamin B_1, plus vitamin D	69.6±2.8	71.6±6.8

[1] Reaction mixture described in text.
[2] Days fed the diet.
[3] Standard error of the mean calculated for 4 rats/group.

B_6 plays a role in the synthesis of coenzyme A (Yamada et al., '56) although this remains to be established on a definite basis. Finally the permeability of cell and mitochondrial membranes cannot be overlooked. Pyridoxine has already been shown to be involved in amino acid transport (Christensen et al., '54) although this is of an active type and not a question of simple permeability. It seems possible that the level of extracellular citrate might well be controlled by membrane phenomena rather than by metabolic means. The effect of vitamin D on citrate oxidation by isolated kidney mitochondria now appears related to changes in the membrane or structural system of these particles (De Luca et al., '60).

Deficiencies of vitamin A and biotin were without definite effect on urinary and tissue citrate (fig. 1d, 1e and table 3). There is a slight trend to higher citrate levels in vitamin A deficiency, but the change is not significant.

In all the experiments dealing with thiamine and biotin very high urinary citrate values were obtained. This was due to the use of egg white instead of casein in these diets. Casein diets supplemented with 0.2% of L-cystine have always given much lower urinary citrate values than egg white diets have.

In general, within each experiment, the effects of the vitamin deficiencies on kidney and serum citrate were similar to those observed in urine. Vitamin D deficiency reduced serum and kidney citrate levels, as was expected from previous results (Steenbock and Bellin, '53). Pantothenic acid and pyridoxine deficiencies also depressed serum and kidney citrate content although in kidney, the depression with the pyridoxine experiment was not great with either vitamin B_6 or vitamin D deficiency. Kidney citrate, like urine citrate levels, was high when egg white diets were fed. In addition, tissue citrate values for rats fed the same diet were noted to vary somewhat from one experiment to another, thereby preventing a critical comparison of absolute citrate values from different experiments. Heart citrate values

TABLE 3

Vitamin deficiency and tissue citrate[1]

Group	Heart	Kidney	Bone	Serum
	μg/gm	*μg/gm*	*mg/gm*	*mg/100 ml*
No vitamin D	22.2 ± 1.3[2]	11.3 ± 1.4[2]	2.72 ± 0.04[2]	2.1 ± 0.1[2]
No pantothenic acid	18.6 ± 2.9	11.9 ± 1.4	5.08 ± 0.20	2.4 ± 0.2
Plus vitamin D, plus pantothenic acid	24.6 ± 1.3	29.3 ± 4.1	5.53 ± 0.27	3.2 ± 0.1
No vitamin D	40.0 ± 3.2	32.8 ± 4.4	—	2.9 ± 0.1
No biotin	35.7 ± 5.6	52.0 ± 5.4	—	2.9 ± 0.3
Plus vitamin D, plus biotin	32.4 ± 5.2	57.7 ± 3.1	—	3.7 ± 0.2
No vitamin D	33.4 ± 4.4	18.8 ± 2.8	2.59 ± 0.34	2.2 ± 0.1
No vitamin B_6	25.2 ± 5.4	17.9 ± 4.2	4.49 ± 0.20	2.4 ± 0.2
Plus vitamin D, plus vitamin B_6	26.7 ± 4.1	24.2 ± 4.5	5.60 ± 0.38	3.0 ± 0.1
No vitamin D	—	31.3 ± 4.1	—	2.2 ± 0.2
No vitamin A	—	51.9 ± 3.6	—	4.0 ± 0.1
Plus vitamin D, plus vitamin A	—	40.0 ± 4.8	—	3.6 ± 0.3
No vitamin D	—	57.0 ± 5.2	—	2.8 ± 0.1
No vitamin B_1	—	82.1 ± 6.8	—	3.8 ± 0.2
Plus vitamin D, plus vitamin B_1	—	77.8 ± 7.3	—	4.4 ± 0.2

[1] Rats were fed their respective diets for 21 days except for the vitamin A (18 days), vitamin B_1 (15 days) and biotin (30 days) experiments. There were 4 to 10 rats in each group.

[2] Standard error of the mean, calculated for 4 rats in each group.

showed no effect of either vitamin D or any of the other vitamins tested. Although biotin deficiency apparently depressed serum citrate and had no effect on urinary citrate (fig. 1) this observation requires further verification before any significance can be attached to it.

Bone citrate values appeared very resistant to change by the vitamin deficiencies except that of vitamin D. Only a slight depression was noted with vitamin B_6 deficiency and little or no depression with pantothenic acid deficiency. The citrate of bone, largely, is not part of the metabolic pool and is found associated with the bone mineral (McLean, '55). Because extracellular levels of citrate were depressed in the two B-vitamin deficiencies, however, it is unexpected to find bone citrate only slightly depressed. It is possible that the citrate of bone is synthesized by cells of the bone which may possess somewhat different metabolic systems or which may be subject to different metabolic controls.

For simplicity, in some experiments data from the animals deficient in both vitamin D and the respective vitamin being studied are not presented; the citrate levels in these instances were always similar to those noted with the animals deficient in vitamin D alone.

SUMMARY

The effect of various vitamin deficiencies on urinary and tissue citrate levels has been compared with that of vitamin D deficiency. Pantothenic acid and vitamin B_6 deficiencies significantly depressed the levels of urinary, serum, and kidney citrate comparable to those found in vitamin D deficiency. They affected bone citrate only slightly, however, whereas vitamin D deficiency reduced this value greatly. Vitamin A and biotin deficiencies were essentially without effect. Although a severe thiamine deficiency depressed urinary citrate, this apparently was due to inanition, because with a mild though definite thiamine deficiency, as indicated by a reduction in pyruvate oxidation by kidney homogenates, urinary and tissue citrate values were unchanged.

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Tissue Lipid Fatty Acid Changes Following the Feeding of High-Cholesterol, Essential Fatty Acid-Supplemented Diets to Rabbits[1]

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The suggested relationship between the essential fatty acids and the genesis of atherosclerosis has received considerable attention in recent years (Sinclair, '56). A number of studies (Luddy et al., '58; Tuna et al., '58; Wright et al., '59; Bottcher et al., '60; Swell et al., '60a, b; Mead and Gouze, '61) have been carried out dealing with the fatty acid composition of the lipid fractions of human tissues, and in particular, serum versus aorta. The several reports are at variance with one another and suggest either an increase, decrease, or no change in the level of polyunsaturated fatty acids in the lipid fractions of the atherosclerotic lesion as compared with serum. Limited information is available regarding the changes occurring in tissue lipid fatty acid composition in animals during the production of experimental atherosclerosis. Fisher et al. ('59) and Fisher and Feigenbaum ('60) reported that the dienoic and tetraenoic fatty acid concentrations of the lipids from the plaque areas were lower than those of the clear areas of the aorta and that aortic fat was more saturated than plasma fat in atherosclerotic chickens. Blomstrand and Christensen ('61) reported that there was a marked increase in the proportion of oleic acid in the cholesterol ester fraction of the aorta of cholesterol-fed cockerels versus the aorta of normal animals. Zilversmit et al. ('61) noted that the cholesterol ester and phospholipid fatty acid composition of plasma and aorta from cholesterol-fed rabbits were markedly different.

Another finding related to the possible role of the essential fatty acids in atherogenesis was made in a recent study (Swell et al., '60c) in which it was shown that the level of arachidonic acid in the serum cholesterol ester fraction of different species was correlated with susceptibility to atherosclerosis. Species which are known to be resistant to atherosclerosis (rat and dog) were observed to have high levels of arachidonic acid ('50 and 17%) in their serum cholesterol ester fraction, whereas species susceptible to the disease, such as the chicken, rabbit, pig, guinea pig, goose and man, had low levels (1.0 to 7.0%). Those findings suggested that susceptibility to atherosclerosis may be related to the ability of each species to synthesize and utilize arachidonic acid. Arachidonic acid is ingested in negligible quantities in the diet and is synthesized in the body from linoleic acid. Pyridoxine may be involved directly or indirectly in that process (Witten and Holman, '52; Swell et al., '61).

Kleinsorge and Zielke ('59) reported that feeding linoleic acid and pyridoxine or arachidonate in a high-cholesterol diet to rats prevented lipid deposition in the aorta. Martens and Hoskins ('58) administered pyridoxine parenterally to rabbits fed a cholesterol-rich diet and did not note any effect of the vitamin in preventing the development of atherosclerosis. The purpose of the present investigation was to ascertain in a species susceptible to atherosclerosis (rabbit), (a) whether arachidonic acid or linoleic acid and pyridoxine could influence the course of atherogenesis, and (b) what changes occur in the fatty acid composition of the tissue lipid fractions during the development of atherosclerosis.

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EXPERIMENTAL

Animals and diets. Zealand white male rabbits weighing 2.5 to 3.0 kg were divided into 4 groups of 5 each and one group of 6. Each of the groups received, in the morning, 50 gm of powdered commercial rabbit pellet chow[2] containing 1 gm of cholesterol with either one of the following: group A, 150 mg of olive oil; group B, 150 mg of linoleic acid; group C, 150 mg of linoleic acid plus 3 mg of pyridoxine hydrochloride in aqueous solution given orally at the time of the morning feeding; and group D, 150 mg of ethyl arachidonate.[3] A control group received 50 gm of powdered pellet chow without additions. The animals consumed their meal in the morning, and in the afternoon were given an additional 75 gm of unground pellet chow. The cholesterol diets were prepared by dissolving 10 gm of cholesterol and 1.5 gm of the fat or fatty acid in 200 ml of ether. This ether solution was added slowly in a mixing bowl to 500 gm of powdered pellet chow. The diet was thoroughly mixed until the ether had evaporated. Preparation of the diet in this manner gave uniform distribution of cholesterol. Water was available to the animals ad libitum. The diets were fed for 9 weeks. At the end of that period the animals were fasted overnight and then sacrificed under pentobarbital sodium anesthesia by exsanguination and the aorta and liver removed. The aorta was carefully cleared of adherent fat, sliced longitudinally and graded for atherosclerosis on a scale from zero to 4 +: zero representing the absence of visible atherosclerosis; 1 +, minimal but visible plaques; 4 +, extensive coverage of most of the aorta with numerous raised confluent plaques; and 2 + and 3 +, intermediate states. Tissues were weighed and extracted according to procedures described earlier (Swell et al., '60b). Free and total cholesterol of the serum and tissue lipid extracts were determined by the method of Sperry and Webb ('50). The cholesterol esters, triglycerides and phospholipids were separated on silicic acid columns and the fatty acid composi-

[2] Purina Rabbit Chow, The Ralston-Purina Company, St. Louis.

[3] Kindly supplied by Hoffman-LaRoche, Inc., Nutley, New Jersey.

TABLE 1

Aortic lipid levels of rabbits fed different diets

Group	Dietary additions	Atherosclerosis[1]	Cholesterol			Triglyceride	Phospholipid
			Free	Ester	Total		
		degree	*mg/gm fresh tissue*				
A	Cholesterol + olive oil	2.5+	1.9 ± 1.0[2]	2.6 ± 1.8	4.5 ± 2.8	40.0 ± 22.0	13.2 ± 2.5
B	Cholesterol + linoleic acid	2.1+	2.3 ± 0.8	2.8 ± 1.8	5.1 ± 2.6	26.5 ± 7.5	14.9 ± 2.3
C	Cholesterol + linoleic acid + vitamin B_6	1.1+	1.7 ± 0.3	1.7 ± 1.2	3.4 ± 1.5	26.4 ± 5.3	20.0 ± 4.1
D	Cholesterol + arachidonic acid	1.6+	2.6 ± 0.4	3.9 ± 1.0	6.5 ± 1.4	32.9 ± 3.9	13.3 ± 1.2
Control		0.0	1.1 ± 0.2	0.1 ± 0.1	1.2 ± 0.2	17.7 ± 3.0	8.5 ± 0.5

[1] Lesions graded grossly from zero to 4+.

[2] Values represent the mean of 5 to 6 rabbits ± standard deviation.

tion of the isolated lipid fractions determined by gas-liquid chromatography as previously described (Swell et al., '60a, b). The triglyceride and phospholipid levels were determined by weighing the appropriate silicic acid column fractions. Statistical analyses were carried out by the *t* test and values of $P < 0.01$ were considered to be significant.

RESULTS

Aorta lipid levels (table 1). The animals of all groups fed cholesterol showed moderate atherosclerosis. On the basis of visual grading, group C, which received the linoleic acid and vitamin B_6 supplement, had the lowest degree of atherosclerosis of the experimental groups. The aortas of all groups showed more than normal amounts of cholesterol; this excess was predominantly in the esterified fraction. Group C had a lower aorta cholesterol level than any of the remaining cholesterol-fed groups. When the cholesterol levels in the aortas of the different experimental groups were compared by statistical tests, however, no significant differences were found. Further studies will be necessary to ascertain whether the linoleic acid and pyridoxine combination in a low-fat diet retards the development of atherosclerosis. There were also increases in the triglyceride and phospholipid levels of the aorta of all experimental groups. No significant differences were found between the levels of those lipid fractions in the aorta of the several groups.

Tissue lipid levels (table 2). At the time of sacrifice all groups had high total blood cholesterol levels. There were no significant differences between the blood cholesterol levels of the experimental groups. The serum triglyceride level showed no significant change from the control value, but an increase occurred in the serum phospholipid level of the cholesterol-fed groups. The liver cholesterol level was high in all experimental groups as compared with the control group; the increase was predominantly in the esterified fraction. The triglyceride concentration was significantly lower in the experimental groups and the liver phospholipid levels did not change as a result of cholesterol feeding.

Serum lipid fatty acids

Cholesterol esters (table 3). Normal rabbit serum cholesterol esters were characterized by a high proportion (39.6%) of linoleic acid (18:2) and a low percentage (1.5%) of arachidonic acid (20:4). Linoleic acid constituted the major fatty acid of the serum cholesterol ester fraction. There was a striking change in the pattern of the serum cholesterol ester fatty acids in the animals with atherosclerosis. A significant decline occurred in the level of linoleic acid, with a corresponding increase in oleic acid when compared with the control group. In groups A to D, oleic acid (18:1) became the major acid (35 to 40%) of the serum cholesterol ester fraction. No significant changes in any

TABLE 2

Serum and liver lipid levels of rabbits fed different diets

Group[1]	Cholesterol			Triglyceride	Phospholipid
	Free	Ester	Total		
			Serum, *mg/100 ml*		
A	322±112[2]	969±372	1291±483	238±82	560±193
B	320±66	873±157	1193±213	168±32	512±58
C	259±67	749±272	1008±333	131±9	391±101
D	435±67	1149±213	1584±276	185±61	517±94
Control	30±10	57±18	87±24	131±29	78±22
			Liver, *mg/gm fresh tissue*		
A	3.9±1.7	10.6±6.4	14.5±9.1	8.6±1.9	32.3±2.8
B	3.9±1.0	10.9±5.2	14.8±6.0	6.7±0.7	31.4±4.9
C	4.7±1.6	13.1±3.8	17.8±5.3	9.1±1.1	30.4±3.9
D	4.3±1.3	12.2±3.5	16.5±4.7	9.0±2.4	30.6±5.2
Control	1.8±0.3	0.6±0.1	2.4±0.4	14.1±2.3	35.3±2.5

[1] See table 1 for description of groups.
[2] Values represent the mean of 5 to 6 rabbits ± standard deviation.

[1] Lesions graded grosaly from zero to 4+.
[2] Values represent the mean of 5 to 6 rabbits ± standard deviation.

of the remaining cholesterol ester fatty acids were noted. The addition of the essential fatty acid supplements (groups B, C and D) did not produce any differences with respect to the cholesterol ester fatty acid spectrum.

Triglycerides (table 4). The triglyceride fatty acids of normal rabbit serum were characterized by a high level of saturated fatty acids (46.8%), of which the major one was palmitic acid (16:0). The polyunsaturated fatty acids (27.0%) and the mono-unsaturated fatty acids (26.2%) were present in the same amounts. The serum triglyceride fatty acids of the several groups were very close in composition to that of the control animals. The only group which showed a change as compared with the control was group C (linoleic acid and pyridoxine) which had a lower percentage of linoleic acid. This difference was found to be significant.

Phospholipids (table 5). Normal rabbit serum phospholipids were characterized by

TABLE 3

Serum cholesterol ester fatty acids of rabbits fed different diets

Fatty acid[1]		Group[2]				
Chain length carbons	No. double bonds	Control	A	B	C	D
		% Total fatty acids				
6 to 15		3.1±1.1[3]	2.1±0.9	2.1±0.5	2.3±0.5	2.1±0.5
16	0	21.3±2.1	20.5±2.2	18.3±2.4	20.3±3.6	22.2±2.4
16	1	4.7±2.4	5.4±0.9	4.4±1.2	5.3±0.9	5.0±0.8
16	2	0.5±0.2	0.6±0.3	0.7±0.2	0.7±0.1	0.7±0.1
17	0	1.1±0.2	1.0±0.2	1.2±0.2	0.9±0.2	1.2±0.2
18	0	3.9±1.3	4.3±1.4	3.1±0.9	4.1±0.8	3.9±0.5
18	1	23.2±5.3	40.6±6.1	38.2±2.4	42.0±3.8	35.2±1.3
18	2	39.6±5.0	22.3±4.8	27.8±1.9	20.9±2.7	25.4±2.9
18	3	0.7±0.4	1.9±0.3	3.1±0.3	2.3±0.5	3.1±0.9
20	0	0.4±0.1	0.5±0.2	0.4±0.2	0.5±0.1	0.5±0.1
20	4	1.5±0.4	0.8±0.2	0.7±0.2	0.7±0.3	0.7±0.1
Saturated		29.4	28.2	24.9	27.8	29.7
Mono-unsaturated		28.3	46.2	42.8	47.6	40.4
Polyunsaturated		42.3	25.6	32.3	24.6	29.9

[1] Represents the principal fatty acids determined; small amounts of others were also detected.
[2] See table 1 for description of groups.
[3] Values represent the mean of 5 to 6 rabbits ± standard deviation.

TABLE 4

Serum triglyceride fatty acids of rabbits fed different diets

Fatty acid[1]		Group[2]				
Chain length carbons	No. double bonds	Control	A	B	C	D
		% Total fatty acids				
6 to 15		2.5±1.4[3]	3.0±0.8	5.0±1.3	3.5±0.7	4.0±1.4
16	0	38.0±2.6	41.3±3.1	38.4±3.8	40.1±2.5	39.8±1.1
16	1	2.6±1.0	2.5±0.4	3.2±0.9	3.5±0.8	2.9±0.8
16	2	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1
17	0	0.6±0.2	0.7±0.2	0.8±0.2	0.8±0.3	0.8±0.4
18	0	5.6±1.9	6.8±1.6	4.5±0.9	5.8±1.1	5.7±1.5
18	1	23.4±2.7	25.8±4.1	24.6±2.9	28.7±3.6	25.7±1.7
18	2	25.2±4.9	18.0±5.1	20.7±2.5	15.4±2.8	18.8±1.9
18	3	1.1±0.3	1.2±0.4	1.8±0.3	1.3±0.3	1.2±0.4
20	0	0.3±0.1	0.2±0.1	0.4±0.2	0.3±0.1	0.4±0.1
20	4	0.5±0.2	0.3±0.1	0.4±0.1	0.4±0.2	0.5±0.1
Saturated		46.8	51.8	48.9	50.3	50.5
Mono-unsaturated		26.2	28.5	28.0	32.4	28.8
Polyunsaturated		27.0	19.7	23.1	17.3	20.7

[1,2,3] See footnotes to tables 1 and 3.

a high percentage of saturated fatty acids (52.3%) and a small amount of polyunsaturated fatty acids (21.2%). The major fatty acid of that serum fraction was palmitic acid (32.9%). With the development of atherosclerosis there was a significant increase in the proportion of linoleic acid in the serum phospholipid fraction of all experimental groups and a drop in the proportion of oleic acid. Group D which received the arachidonate supplement had a significantly greater amount of arachidonic acid in its serum phospholipid fraction than any of the other experimental groups.

Aorta lipid fatty acids

Cholesterol esters (table 6). Normal rabbit aorta contained only traces of cholesterol ester and therefore it was not possible to obtain adequate amounts for fatty acid analysis; the values reported in table 6 represent those esters deposited in the aorta as a result of cholesterol feeding.

TABLE 5

Serum phospholipid fatty acids of rabbits fed different diets

Fatty acid[1]		Group[2]				
Chain length carbons	No. double bonds	Control	A	B	C	D
		% *Total fatty acids*				
6 to 15		2.0±0.7[3]	1.5±0.3	1.9±0.3	1.7±0.9	1.4±0.5
16	0	32.9±3.0	36.5±3.1	37.2±4.9	37.5±4.2	40.5±2.0
16	1	1.2±0.3	1.5±0.5	1.4±0.4	1.6±0.4	1.1±0.4
17	0	1.5±0.5	1.3±0.4	1.3±0.4	1.0±0.3	1.1±0.5
18	0	15.1±4.5	15.2±1.9	12.9±2.7	12.7±2.4	13.8±1.4
18	1	25.3±3.4	15.0±2.0	12.9±1.1	15.1±2.6	13.8±0.7
18	2	15.6±2.5	24.0±3.0	27.5±2.0	24.9±2.3	20.9±3.3
18	3	0.3±0.1	0.4±0.2	0.2±0.1	0.3±0.1	0.3±0.1
20	0	0.8±0.2	0.5±0.3	0.5±0.2	0.7±0.1	0.6±0.2
20	4	2.4±0.9	1.5±1.0	1.4±0.8	1.5±0.5	3.2±1.0
22[4]		2.9±0.8	2.6±0.5	2.8±0.5	3.0±0.6	3.3±0.7
Saturated		52.3	55.0	53.8	53.6	57.4
Mono-unsaturated		26.5	16.5	14.3	16.7	14.9
Polyunsaturated		21.2	28.5	31.9	29.7	27.7

[1,2,3] See footnotes to tables 1 and 3.
[4] Represents long-chain polyunsaturated fatty acids, 22 carbons and longer.

TABLE 6

Aorta cholesterol ester fatty acids of rabbits fed different diets

Fatty acid[1]		Group[2]			
Chain length carbons	No. double bonds	A	B	C	D
		% *Total fatty acids*			
6 to 15		3.9±1.7[3]	3.5±1.5	4.8±1.9	3.2± 0.7
16	0	31.0±6.5	29.6±5.6	38.1±8.5	36.2±11.1
16	1	4.1±1.1	2.9±1.0	3.0±0.9	3.5± 0.7
16	2	1.4±0.9	1.3±0.6	1.6±0.6	1.2± 0.3
17	0	0.2±0.1	0.2±0.1	0.2±0.1	0.2± 0.1
18	0	6.7±1.2	5.2±1.6	7.9±2.7	4.8± 0.8
18	1	39.4±7.9	39.8±5.4	33.6±7.6	36.8± 7.7
18	2	11.8±3.4	15.3±4.5	8.0±3.3	11.9± 4.0
18	3	0.7±0.2	1.0±0.5	1.0±0.4	1.2± 0.4
20	0	0.5±0.2	0.8±0.3	0.7±0.3	0.6± 0.3
20	4	0.3±0.1	0.4±0.1	1.1±0.6	0.4± 0.2
Saturated		42.3	39.1	51.5	44.8
Mono-unsaturated		43.5	42.9	36.8	40.5
Polyunsaturated		14.2	18.0	11.7	14.7

[1,2,3] See footnotes to tables 1 and 3.

There were no significant differences in the cholesterol ester fatty acid composition between the several experimental groups. The aorta cholesterol ester fatty acids of the experimental groups were characterized by a high percentage of saturated and mono-unsaturated fatty acids which occurred in approximately equal proportions and a very low level of polyunsaturated fatty acids (12 to 18%). The major acids were palmitic and oleic acids which were present in approximately equal quantities. The arachidonic acid in this fraction was exceedingly low (0.3 to 1%).

Triglycerides (table 7). Normal rabbit aorta triglyceride fatty acids consisted predominantly of palmitic, oleic and linoleic acids. Cholesterol feeding did not alter the aorta triglyceride fatty acid composition.

Phospholipids (table 8). The phospholipid fatty acids of normal rabbit aorta had a high proportion of saturated fatty acids (56.6%) and a low percentage of polyunsaturated fatty acids (14.0%). Cholesterol feeding did not alter the aorta phospholipid fatty acid composition.

TABLE 7

Aorta triglyceride fatty acids of rabbits fed different diets

Fatty acid[1]		Group[2]				
Chain length carbons	No. double bonds	Control	A	B	C	D
		% *Total fatty acids*				
6 to 15		3.1±1.1[3]	4.3±1.4	4.0±1.2	3.3±0.8	4.1±1.2
16	0	38.8±4.8	37.6±2.8	39.0±3.1	43.2±2.9	37.4±2.2
16	1	2.9±0.8	3.2±0.7	3.1±0.5	3.5±0.8	3.6±1.4
16	2	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1
17	0	0.5±0.2	0.3±0.1	0.2±0.1	0.2±0.1	0.2±0.1
18	0	5.2±0.6	5.4±0.2	5.7±1.2	5.6±0.7	5.3±0.8
18	1	28.1±3.6	24.8±2.1	25.9±2.0	27.5±1.3	24.0±1.7
18	2	19.1±4.7	22.0±4.1	19.9±2.7	14.9±4.4	22.9±1.3
18	3	1.5±0.6	1.5±0.7	1.5±0.8	1.0±0.1	1.7±0.3
20	0	0.3±0.1	0.5±0.2	0.3±0.1	0.3±0.1	0.3±0.1
20	4	0.3±0.1	0.2±0.1	0.2±0.1	0.3±0.1	0.3±0.2
Saturated		47.9	47.9	49.0	52.4	47.1
Mono-unsaturated		31.0	28.2	29.2	31.2	27.8
Polyunsaturated		21.1	23.9	21.8	16.4	25.1

[1,2,3] See footnotes to tables 1 and 3.

TABLE 8

Aorta phospholipid fatty acids of rabbits fed different diets

Fatty acid[1]		Group[2]				
Chain length carbons	No. double bonds	Control	A	B	C	D
		% *Total fatty acids*				
6 to 15		2.0±0.3[3]	2.5±1.0	2.8±1.0	3.4±1.4	2.3±0.6
16	0	42.3±2.5	41.5±5.0	41.0±3.8	47.0±8.4	45.0±4.4
16	1	1.3±0.3	1.8±0.6	1.6±0.5	2.3±0.8	1.6±0.8
17	0	0.5±0.4	0.5±0.2	0.6±0.2	1.0±0.5	0.5±0.2
18	0	11.1±1.5	14.0±1.5	13.1±1.7	9.0±1.8	10.2±3.0
18	1	28.1±4.4	27.7±5.8	27.8±3.7	27.7±5.5	29.2±1.8
18	2	9.1±3.2	7.6±2.1	8.7±2.2	5.3±2.8	6.0±1.8
18	3	0.7±0.3	0.4±0.1	0.8±0.4	0.6±0.3	0.9±0.4
20	0	0.7±0.3	0.9±0.5	0.5±0.4	0.4±0.2	0.7±0.2
20	4	1.7±0.8	0.8±0.3	0.6±0.1	0.9±0.2	0.9±0.4
22[4]		2.5±1.0	2.3±0.9	2.5±0.5	2.4±0.4	2.7±0.6
Saturated		56.6	59.4	58.0	60.8	58.7
Mono-unsaturated		29.4	29.5	29.4	30.0	30.8
Polyunsaturated		14.0	11.1	12.6	9.2	10.5

[1,2,3,4] See footnotes to tables 1, 3 and 5.

Serum versus aorta lipid fatty acids

Cholesterol esters. The fatty acid composition of the cholesterol ester fraction of the serum (table 3) was different from that fraction of the aorta (table 6) in the several groups. The serum cholesterol ester fraction of all groups had significantly more linoleic acid and less palmitic acid than that fraction in the aorta. These differences were more pronounced in the control group than in the cholesterol-fed groups.

Triglycerides. The triglyceride fatty acid composition of the serum (table 4) and aorta (table 7) were very similar in all groups.

Phospholipids. Although there was an increase in the amount of linoleic acid in the serum phospholipid fraction (table 5) of the animals receiving the high cholesterol diets, there was no marked change in the aorta phospholipid fatty acid composition (table 8). That fraction in the serum and the aorta of the control animals was

TABLE 9

Liver cholesterol ester fatty acids of rabbits fed different diets

Fatty acid[1]		Group[2]				
Chain length carbons	No. double bonds	Control	A	B	C	D
		% *Total fatty acids*				
6 to 15		2.4 ± 0.7[3]	2.3 ± 0.7	2.5 ± 0.8	2.0 ± 0.6	2.3 ± 0.9
16	0	28.3 ± 2.2	25.3 ± 2.7	22.5 ± 1.4	23.1 ± 3.1	22.6 ± 2.6
16	1	2.9 ± 0.5	3.3 ± 0.8	5.2 ± 1.7	6.7 ± 1.9	4.6 ± 1.0
16	2	0.2 ± 0.1	0.2 ± 0.1	1.0 ± 0.3	0.7 ± 0.3	0.7 ± 0.2
17	0	1.1 ± 0.1	1.1 ± 0.2	1.5 ± 0.1	1.1 ± 0.2	1.0 ± 0.2
18	0	18.0 ± 4.8	9.2 ± 2.1	6.2 ± 1.4	5.3 ± 0.7	6.0 ± 0.9
18	1	29.4 ± 3.6	40.3 ± 3.8	42.8 ± 3.8	47.1 ± 4.9	44.5 ± 5.8
18	2	15.2 ± 2.6	15.8 ± 1.2	15.2 ± 1.4	11.8 ± 2.3	15.3 ± 4.9
18	3	1.0 ± 0.2	1.2 ± 0.3	1.7 ± 0.3	1.2 ± 0.4	1.4 ± 0.5
20	0	0.7 ± 0.2	0.5 ± 0.2	0.6 ± 0.2	0.5 ± 0.1	0.6 ± 0.1
20	4	0.8 ± 0.3	0.8 ± 0.4	0.8 ± 0.2	0.5 ± 0.1	1.0 ± 0.5
Saturated		50.3	37.9	32.8	31.7	32.1
Mono-unsaturated		32.5	44.1	48.5	54.1	49.5
Polyunsaturated		17.2	18.0	18.7	14.2	18.4

[1,2,3] See footnotes to tables 1 and 3.

TABLE 10

Liver triglyceride fatty acids of rabbits fed different diets

Fatty acid[1]		Group[2]				
Chain length carbons	No. double bonds	Control	A	B	C	D
		% *Total fatty acids*				
6 to 15		2.4 ± 0.6[3]	3.0 ± 0.9	4.5 ± 0.7	4.5 ± 1.6	3.2 ± 0.5
16	0	37.6 ± 2.6	43.4 ± 2.6	41.4 ± 5.2	40.4 ± 3.2	46.0 ± 1.7
16	1	2.0 ± 0.4	3.1 ± 1.1	4.1 ± 0.8	4.8 ± 1.0	3.1 ± 0.6
16	2	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
17	0	0.6 ± 0.1	0.7 ± 0.1	1.1 ± 0.4	0.6 ± 0.2	0.6 ± 0.1
18	0	4.4 ± 0.8	4.5 ± 0.5	4.8 ± 1.3	5.2 ± 1.9	5.0 ± 1.8
18	1	20.5 ± 2.1	26.0 ± 4.9	28.4 ± 4.2	31.2 ± 3.1	25.2 ± 4.7
18	2	30.2 ± 3.4	17.5 ± 3.7	13.7 ± 1.7	11.0 ± 2.3	15.1 ± 4.5
18	3	1.5 ± 0.3	0.8 ± 0.3	0.8 ± 0.2	0.9 ± 0.2	0.9 ± 0.2
20	0	0.3 ± 0.1	0.4 ± 0.1	0.6 ± 0.2	0.6 ± 0.4	0.5 ± 0.3
20	4	0.3 ± 0.1	0.4 ± 0.2	0.4 ± 0.1	0.6 ± 0.3	0.2 ± 0.1
Saturated		45.1	51.8	52.0	50.9	55.1
Mono-unsaturated		22.7	29.3	32.9	36.4	28.5
Polyunsaturated		32.2	18.9	15.1	12.7	16.4

[1,2,3] See footnotes to tables 1 and 3.

also different in fatty acid composition. The changes occurring in the serum fraction may reflect the greater abundance of a specific phospholipid.

Liver lipid fatty acids

Cholesterol esters (table 9). The cholesterol ester fraction of the liver of all groups was characterized by a low percentage of polyunsaturated fatty acids, and in particular, linoleic acid. No significant differences were observed in the liver cholesterol ester fatty acid composition among the groups fed the cholesterol and fatty acid-supplemented diets. There was, however, a significant reduction in the proportion of stearic acid (18:0) and an increase in the level of oleic acid in the experimental groups as compared with the control animals.

Triglycerides (table 10). The liver triglyceride fraction of the several cholesterol-fed groups had significantly less linoleic acid than that of the control animals. That decline in linoleic acid was made up by proportionate increases in the levels of oleic and palmitic acids.

Phospholipids (table 11). The liver phospholipid fraction showed the most marked differences with respect to the effect of the essential fatty acid-supplemented diets. The most notable effect on the fatty acid composition was in group D which received arachidonate. Group D had significantly more arachidonic acid and linoleic acid than groups A, B and C. Group C which received the linoleic acid and vitamin B_6 supplement also showed a higher proportion of linoleic acid than groups A and B, although not as high as group D which received arachidonate.

DISCUSSION

In interpreting the results of the present study the following points are pertinent. Ethyl arachidonate fed at a level of 150 mg per day was a large amount in terms of the total body arachidonic acid level of the rabbit. The addition of the small amounts of fatty acids (150 mg), however, did not substantially alter the proportion of fat in the diets of the animals. The rabbit pellet chow fed was previously analyzed and contained only 2.4% of fat (Swell et al., '60c). Thus, the changes occurring in the tissue lipid fatty acids reflect the effect of a high-cholesterol low-fat diet. The feeding of arachidonate did not alter the course of atherosclerosis, nor, with the exception of the phospholipid fraction in the serum and liver, were there any elevations in the percentage of arachidonate in any of the tissue lipid fractions when compared with the other groups fed the high-cholesterol,

TABLE 11

Liver phospholipid fatty acids of rabbits fed different diets

Fatty acid[1]		Group[2]				
Chain length carbons	No. double bonds	Control	A	B	C	D
		% *Total fatty acids*				
6 to 15		0.9±0.5[3]	1.5±0.5	2.1±0.4	1.8±0.7	1.2±0.5
16	0	23.5±4.2	35.0±8.8	40.4±4.7	35.0±8.8	29.6±3.2
16	1	1.0±0.5	1.8±0.8	1.7±0.6	2.1±0.6	1.2±0.1
16	2	0.2±0.1	0.2±0.1	0.2±0.1	0.5±0.2	0.2±0.1
17	0	1.0±0.3	2.7±1.5	1.5±0.4	1.5±0.6	0.7±0.1
18	0	25.7±3.2	23.0±4.3	22.8±1.1	15.2±6.7	17.0±1.5
18	1	9.6±3.5	15.9±4.7	15.4±2.4	18.0±3.7	13.6±1.7
18	2	26.6±6.1	11.1±2.9	8.4±3.0	16.4±3.4	23.0±1.0
18	3	0.6±0.2	0.6±0.3	0.5±0.1	0.7±0.2	0.6±0.1
20	0	0.3±0.1	0.3±0.1	0.4±0.1	0.5±0.3	0.4±0.1
20	3	0.2±0.1	0.2±0.1	0.2±0.1	0.3±0.1	0.2±0.1
20	4	4.5±1.0	2.4±1.1	1.7±0.3	2.6±1.7	5.8±2.7
22[4]		6.0±1.5	5.3±1.1	4.7±0.9	5.4±1.5	6.5±2.0
Saturated		51.2	62.3	67.0	53.8	48.7
Mono-unsaturated		10.8	17.9	17.3	20.3	15.0
Polyunsaturated		37.9	19.8	15.7	25.9	36.3

[1,2,3,4] See footnotes to tables 1, 3 and 5.

fatty acid-supplemented diets. It thus appears, that, even though a relatively large amount of arachidonate was fed, the composition of the tissue lipid fractions was not appreciably altered. In view of the previously described relationship (Swell et al., '60c) between arachidonic acid in the serum cholesterol esters and species susceptibility to atherosclerosis, these results suggest that arachidonate synthesis and utilization is well regulated in the rabbit and not readily susceptible to alteration. Such inherent species differences in arachidonate metabolism, and in particular, cholesterol ester metabolism, may provide some important information regarding the pathogenesis of atherosclerosis. None of the remaining diets had any significant effect on the course of the disease nor on the lipid levels in the tissues. The effect of the linoleic acid and pyridoxine diet is equivocal and further studies are needed to ascertain whether such regimens have a beneficial effect. A recent study (Swell et al., '61) has shown that pyridoxine may not play a direct role in arachidonate biosynthesis.

Data on the lipid fatty acid composition of rabbit aorta, serum and liver indicate that distinct changes occur in tissue lipid fatty acid composition as a result of cholesterol feeding. The most notable changes occurred in the serum and tissue cholesterol ester fractions. The cholesterol esters of the aorta in all groups with atherosclerosis showed a significantly larger proportion of saturated and oleic acids and significantly less linoleic acid than that fraction in the serum of normal animals. These observations are in agreement with our earlier reports in man (Swell et al., '60a, b) and provide added support for the concept that a derangement of essential fatty acid metabolism, and, in particular, of cholesterol ester metabolism may be an important factor in the etiology of atherosclerosis. The changes in the serum and tissue phospholipid fatty acid composition are difficult to interpret because the phospholipid fraction is heterogenous and changes may have occurred in only one of the phospholipid fractions. However, while the polyunsaturated fatty acid level in the serum cholesterol ester fraction decreased upon cholesterol feeding, the polyunsaturated fatty caids of the serum phospholipid fraction increased. At the same time, the aorta phospholipid fatty acid composition did not change. These observations may be interpreted to indicate that the aortic phospholipid fraction is derived from synthesis within the tissue in agreement with Zilversmit and McCandless ('59) or that only specific phospholipids are deposited in the aorta from serum.

Observations with respect to the liver cholesterol ester fatty acid composition confirm our earlier findings in the rat (Swell et al., '60d) and man (Swell et al., '60a) and indicate that the liver cholesterol esters of the normal animal contain a much higher proportion of saturated fatty acids than the serum cholesterol ester fraction. The feeding of cholesterol increased the proportion of oleic acid in the liver cholesterol esters. Similar observations have been made by Evans et al. ('59) who fed rabbits cholesterol with tallow. In the cholesterol-fed rabbits, however, the liver cholesterol ester was decidedly different in fatty acid composition from the aorta or the serum. The non-homogeneity of the cholesterol ester fraction in the liver and other tissues further supports the view (Swell et al., '58; Field et al., '60) that serum cholesterol esters may not be directly derived from the liver, but perhaps through a process of exchange with other lipids as well as through direct esterification of cholesterol in other tissues.

SUMMARY

Rabbits were fed a normal stock diet supplemented with 1 gm of cholesterol per day plus either one of the following: 150 mg olive oil, 150 mg linoleic acid, 150 mg linoleic acid plus 3 mg pyridoxine, and 150 mg ethyl arachidonate. A control group fed the stock diet was run in parallel. The animals were sacrificed after 9 weeks, the aorta graded visually and the tissues analyzed for lipids and lipid fatty acid composition by gas-liquid chromatography. All of the cholesterol-fed groups showed moderate degrees of atherosclerosis. There did not appear to be any differences with respect to the effect of the various diets on the development of atherosclerosis with the possible exception of the group receiving linoleic acid and pyridoxine. The cho-

lesterol, triglycerides and phospholipids of the aorta were elevated in all experimental groups above the control. Increases in the liver and serum cholesterol occurred, but no differences were noted among the cholesterol-fed groups. There were marked differences in tissue lipid fatty acid composition between the several cholesterol-fed groups and the control animals. The serum cholesterol ester fraction of the rabbits with atherosclerosis had substantially more oleic acid and less linoleic acid than that fraction in the serum of normal animals. The cholesterol esters deposited in the aorta of the cholesterol-fed groups contained a significantly greater proportion of saturated and oleic acids and significantly less linoleic acid than that fraction in the serum of normal animals. No significant differences occurred in the fatty acid composition of the aorta triglyceride and phospholipid fractions between normal and atherosclerotic animals. In the animals with atherosclerosis, however, there was an increase in the proportion of linoleic acid in the serum phospholipid fraction. The group fed ethyl arachidonate had significantly more of that acid in the liver phospholipid fraction than the other fatty acid-supplemented groups. The results of this study provide further evidence that the essential fatty acids may play an important role in the pathogenesis of atherosclerosis, and in particular that arachidonic acid and cholesterol ester metabolism require further investigation.

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Growth and Dietary Pattern of Rats Fed Self-Selection Diets Following Whole-Body Irradiation[1]

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Animals living in their natural environment instinctively select diets adequate enough to maintain growth and reproduction. The laboratory rat also has demonstrated the ability to select an adequate diet from a variety of nutrients offered in separate containers (Osborne and Mendel, '18; Richter et al., '38).

Cravings for various foods by animals are often attempts to overcome certain nutritional deficiencies or disorders. For example, rats supplied with diets deficient in either thiamine, riboflavin, or pyridoxine will tend to select those diets containing the respective vitamins (Scott and Quint, '46). Increased nutritional demands during pregnancy and lactation also elicit abnormal cravings for calcium and protein (Richter and Barelare, '38).

The present study was designed to investigate further the apparent ability of rats to voluntarily select a diet according to the needs of the body and to detect possible metabolic derangements or deficiencies resulting from exposure to moderate doses of x-irradiation.

EXPERIMENTAL

Male rats of the Sprague-Dawley strain were obtained at weanling age from the Naval Radiological Defense Laboratory's (NRDL) pathogen-free colony and used in this study. Each of the 44 rats was individually housed and fed in large, wire-bottom cages. All animals initially were fed a basal diet composed of the following in per cent: crude casein, 24; corn oil, 10; sucrose, 57; dried brewer's yeast, 4; mineral mix,[2] 4; and cod liver oil, 1. Following a 14-day adjustment period, 28 rats were placed on a self-selection regimen and the remaining 16 rats continued to be fed the basal diet. The rats on the self-selection regimen were offered the above ingredients and distilled water ad libitum in separate containers except the corn oil and cod liver oil which were combined in a 10:1 ratio. The dry ingredients were offered in pint-size, wide-mouth mason jars that were converted to feeding containers. The corn oil-cod liver oil and distilled water were offered in glass vials and bottles, respectively, with glass drinking tubes. The jars and bottles were rotated in the cages every other day in a random, predetermined manner in one-half of the rats supplied with self-selected diets. The rats receiving basal diets also were allowed ad libitum quantities of the basal, premixed diet and distilled water. Daily measurements of food and water intakes and body weights were obtained.

Following a 21-day period on the experimental regimen, all the rats receiving self-selected diets and one-half of the rats maintained with the basal diet were exposed to a single dose of 375-rad x-irradiation[3] and three weeks later, to a second 375-rad dose.

The relative proportions of nutrients consumed were converted to calories using the average physiological fuel values of 4, 9, and 4 Cal. per gm of protein, fat and carbohydrate, respectively.

RESULTS

During the pre-irradiation period, two rats obviously were unable to select an adequate diet; and two other rats died quite

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[1] The opinions and assertions contained herein are those of the author and are not to be construed as official or reflecting the views of the Navy Department.

[2] Salt Mixture, H.M.W., Nutritional Biochemicals Corporation, Cleveland.

[3] X-rays, 250 kvp, 15 ma, filters; 0.5 mm Cu + 1 mm Al (HVL, 1.4 mm Cu), target distance, 40 inches; dose rate, 28 rad per minute.

TABLE 1

Variability in average daily food intake of an individual rat and in a group of rats on self-selection diets during pre-irradiation period[1]

Animals	No. of animals	Diet ingredient				
		Casein	Yeast	Sucrose	Fat	Minerals
		gm/day	*gm/day*	*gm/day*	*gm/day*	*gm/day*
Individual	1	3.0±0.8	1.3±0.9	13.3±1.8	0.1±0.1	0.4±0.4
Range		(2.0–5.0)	(0–3.0)	(10.0–16.0)	(0–0.4)	(0–1.0)
Group	18	4.0±2.0	3.6±1.5	6.8±3.0	1.0±1.0	0.2±0.2
Range		(1.1–7.5)	(1.4–6.5)	(1.4–11.5)	(0.1–3.7)	(0.1–0.7)

[1] Including standard deviation.

suddenly of unknown causes and are, therefore, excluded from the results to be presented. The remaining 24 rats on the self-selection regimen were able to select a diet that resulted in growth equal to that of rats fed the basal diet. Rotation of the food and water containers did not influence the selections or growth rates. Statistical analysis of growth rates, total food intake and efficiences of food utilization prior to the irradiation showed no significant differences between rats fed basal or self-selected diets. In general, the patterns of food intake were quite variable between rats but relatively consistent for any given animal, particularly for the casein and sucrose, as shown in table 1.

Within 4 days after the initial 375-rad x-irradiation, 4 rats had died. Gross findings upon autopsy indicated that two died with urethral proteinaceous plugs and two with internal hemorrhaging. By the 14th day post-irradiation, two additional rats died, presumably from the effects of radiation. All rats that died had been supplied with self-selected diets and 5 of the 6 were, at one time or another, high-fat consumers, selecting as much as 75% of their total calories as fat. The apparent appetite for fat, however, may have reflected a dislike for casein or the other ingredients.

The mean differences in body weight between the two irradiated groups and the nonirradiated control group fed the basal diet are illustrated in figure 1. As shown, rats fed the basal diet lost significantly more body weight after both radiation doses than the rats that selected their own diet. It required 7 days for the rats receiving the self-selection diet and 12 days for those supplied with the premixed diet to regain the weight observed at the time of the first radiation. The differences in body weight are not attributable entirely to the variance in weight of water consumed between the two groups since they never approached that of body weight differences (fig. 2). The apparent polydipsia depicted in figure 2, one-day post-irradiation, was due primarily to excessive intakes by one or two rats in each group; otherwise, the intake of water was similar before and after the exposure.

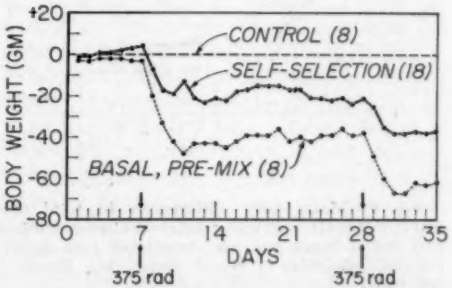

Fig. 1 Mean daily differences in body weight between control rats fed a basal diet and irradiated rats supplied with self-selection or basal diets. Zero reference line indicates non-irradiated, basal diet; solid line, irradiated, self-selection diet; dotted line, irradiated, basal diet. Number of animals per group in parentheses.

The relatively uniform daily food intakes by the nonirradiated group fed the basal diet permitted daily comparisons of nutrient intake and subsequent patterns of intake between the various groups. The relative proportions of nutrient intake expressed in calories are shown in figures 2 and 3. During the pre-irradiation period, the intakes by rats receiving self-selection diets approximated the levels of intake

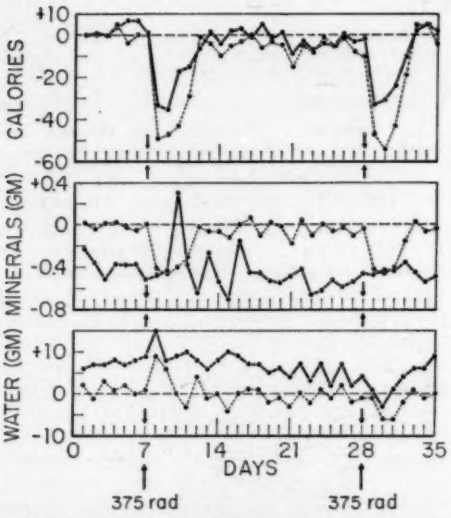

Fig. 2 Mean daily differences in total calories, minerals and water intake between control rats fed a basal diet and irradiated rats receiving self-selection or basal diets. See figure 1 for code.

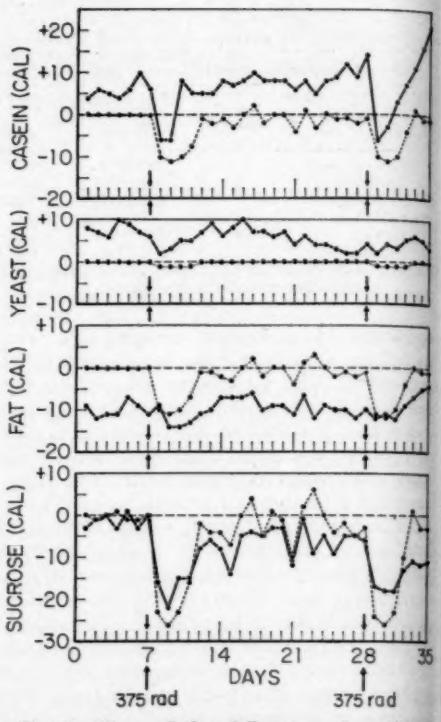

Fig. 3 Mean daily differences in calories from casein, yeast, fat and sucrose between control rats fed a basal diet and irradiated rats supplied with self-selection or basal diets. See figure 1 for code.

of the control group only in total calories and calories from sucrose. The intakes of casein, yeast and water were greater, whereas fat (corn oil-cod liver oil) and minerals were considerably below the levels fed rats supplied with premixed diets. After the first irradiation, selection and intake of casein returned to pre-irradiation levels by the third day (fig. 3) and continued to increase during the post-irradiation period. Following the second x-ray dose, the intake again increased subsequent to the initial drop immediately after the irradiation.

The selections of yeast and corn oil remained essentially unchanged during the post-irradiation period. The selection and intake of sucrose following irradiation decreased, however, and never returned to pre-exposure levels. Similar results were noted after the second exposure.

The mean total caloric intakes of the irradiated rats receiving basal and self-selected diets returned to pre-irradiation levels by the 5th day (fig. 2). The mean caloric intake of the rats fed the self-selection diet immediately after the x-ray was greater than similarly treated rats receiving the basal diet. On the third day after irradiation, the difference was over 20 Cal., suggesting that the rats allowed free choice selections of the diet ingredients were either recovering from the anorectic response sooner or their appetite, particularly for protein, was enhanced, or both.

The intake of salts also increased on the third day after the first irradiation from an average of 0.24 to 1.25 gm per rat per day, or over 5 times the pre-irradiation level. The high intake was not paralleled by a concomitant increase in water intake. The mineral intake was not modified after the second exposure (fig. 2).

DISCUSSION

If an animal can voluntarily select a diet pattern conducive to normal growth

then, conversely, if normal growth occurs, the diet selected must be an adequate one. The majority of the rats in this study exhibited normal growth and thus, apparently were able to select an adequate diet. As expected, some of the rats were unable to make adequate selections due, in part, to the limited number of foods offered and to unpalatability of one or more of the ingredients. Scott ('46) also noted that many rats failed to select an adequate diet when offered a limited number of purified foods. He also observed wide variabilities in appetite between rats as in the present study (table 1). This characteristic prevented the calculation of meaningful averages and standard errors of nutrient intake in the present study but allowed the delineation of average intake patterns. This was accomplished by: (a) converting the intakes of each ingredient to calories; (b) depicting the relative proportions of intake for a group as daily differences from a control group; and (c) by including control values obtained prior to the irradiation, thus, allowing intragroup comparisons. Then, if the general trend of a pattern is modified subsequent to a given stress, the resultant change may reflect certain metabolic derangements or imbalances. Such a change was noted with the mineral and casein intakes. The high intake of minerals on the third day after the first irradiation suggested an increased craving probably aggravated by excess electrolyte loss from the body. This is in accordance with the observations of Jackson et al. ('58), who noted that the excretion of potassium and sodium increased on the third day after irradiation in fasting rats. It is recognized, however, that the apparent craving in the present study may not represent appetite for either sodium or potassium alone, since the mineral mix also contained other salts. It is perhaps significant that the minerals were voluntarily selected even though the salt mixture was composited for inclusion in a mixed diet and not necessarily for isolated consumption.

The slight and gradual but sustained increase in the intake of casein following irradiation may represent greater demands for protein as growth progressed although such an increase was not observed in similar studies by Richter and Barelare ('39). The relatively early preference for casein in this study was of interest and may be indicative of an early attempt to replenish loss of protein after the irradiation. That losses do occur after irradiation was clearly shown in studies by Gustafson and Koletsky ('52) with rats exposed to 660 r x-irradiation and was presumed to be due, in part, to tissue destruction and to probable alteration in metabolism. At any rate, the increase in casein intake would be consistent with experimental evidence that high levels of protein facilitate regeneration of damaged tissues, particularly in situations of concurrent calorie deficit (Pollack and Halpern, '52).

The early selection of casein also may be related to the variation in body weight between the rats supplied with self-selected and premixed diets. Another factor to be considered is the greater total intake of protein by the rats selecting their own diet. It is not unreasonable to suppose that under conditions of high-protein intake, loss of weight following irradiation may be modified to some extent by virtue of differences in excretion and retention of water. That rats consume greater quantities of water when fed high-protein diets is well known (Maynard and Loosli, '56) and also was observed in the present study (fig. 2). Although an increase in water intake is normally associated with a corresponding increase in excretion, this may not occur in the irradiated animal. The subject invites further investigation with particular attention to the evaluation of weight loss and recovery in rats fed high-protein diets and when fasted for various periods after exposure to a moderate dose of x-irradiation.

The results of this study indicate the applicability of allowing rats free-choice selections of their diets to demonstrate possible metabolic derangements or imbalances following irradiation as revealed by their subsequent patterns of nutrient intake. The technique should also be considered in other studies where nutritional disorders are suspected concomitant with disease.

SUMMARY

The apparent ability of rats to voluntarily select a dietary according to the needs of the body was used in this study to detect possible metabolic derangements subsequent to a dose of 375-rad whole-body x-irradiation. The animals were allowed free choice selections of various foodstuffs offered in separate containers and the relative amounts of food intake as well as body weights were measured daily.

The majority of the rats were able to select diets sufficiently adequate to support normal growth. Upon irradiation at the level used, rats fed a basal premixed diet lost significantly more body weight than similarly treated rats selecting their own diet. In the overall pattern, the proportion of casein selected increased progressively, whereas that of sucrose decreased subsequent to the irradiation. The selections of yeast and corn oil remained essentially the same. On the third day only, after irradiation, the intake of minerals increased to over 5 times the pre-irradiation level. The selection and intake of casein returned to pre-irradiation levels by the third day, whereas the total caloric intake of irradiated rats fed basal and self-selected diets returned to normal levels by the 5th day. Possible explanations for the apparent craving for casein and minerals after the level of irradiation used are discussed briefly.

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Length of Life and Nutrition in the Rat[1]

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A longevity study of the rat was undertaken to test the concept that enzymatic activity levels and patterns are related to aging processes, and that these patterns are modifiable by nutritional means. This concept further evisions that these modified enzyme patterns result in changing incidences of organic disease and in changing patterns of mortality. Data obtained during the course of this longevity study, which will be presented in successive papers, relate nutritional state and chronological age to growth, senescence, biochemical patterns (particularly hepatic enzyme activity), hepatic cytological changes and the development, incidence and severity of organic disease.

This paper will present data allowing an assessment of the effect of uniform lifelong dietary regimens upon length of life. Four semisynthetic diets were fed on both a restricted and an unrestricted basis and were designed to define the influence upon the parameters studied during lifelong feeding of precise amounts of casein and of sucrose in otherwise adequate diets.

MATERIALS AND METHODS

Animals and animal care. The more than 1000 male weanling rats used in two series of lifetime studies were purchased from the Charles River Breeding Laboratories. The Charles River SD rats (Sprague-Dawley descendants) were selectively bred for rapidity of growth and relative freedom of disease. All breeding rats were routinely vaccinated for *Salmonella enteriditis* and *S. typhomurium*.

Inasmuch as the environment of a large animal population may have definite bearing upon the data obtained in long-term studies, handling, housing and maintenance will be described in detail. Each cage, housing one animal, was of the sliding drawer type, made of galvanized metal with wire-mesh bottom and front, measuring 7 × 7 × 9 inches. An 8-ounce inverted bottle with glass tip was mounted on the front of the cage for water and a food bottle was secured by a metal band within the cage to prevent tipping. Five-foot high, mobile racks accepting 30 cages to a side in rows of 6 were used to hold cages securely and maintain their separation.

All rats were housed in one room which afforded more than 10 cubic feet of air space per animal. Temperature was maintained between 73 to 75°F by 4 air conditioners and two dehumidifiers in warm weather, and by 5 hot water radiators in colder weather. Circulation of air was maintained by an exhaust system and a battery of continuously running, small-bladed fans placed near the ceiling. Daylight entered the room through large, frosted-glass windows and artificial light was supplied only during caretaker working hours (8 AM to 5 PM) by fluorescent lamps and fixtures suspended from the ceiling. Seven ultra-violet lamps were evenly spaced in the room, and continuously irradiated the ambient air above cage level but did not irradiate the animals directly.

Unnecessary noise was avoided and traffic by personnel not directly associated with this project was restricted. At intervals, cage position was changed within the room. Housekeeping chores were performed daily and cages and water bottles were washed when necessary.

All rats were delivered by the supplier at exactly 21 days of age and, upon arrival, were weighed, examined for well-being and assigned individual cages. Thereafter, all rats were weighed to the nearest gram at weekly intervals, at the same time of day, and examined for the

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presence of external and possible internal abnormalities. Handling of all animals in all groups was the same. No therapeutic measure was undertaken for any animal other than the shortening of excessively long incisors.

Postmortem examination and food preparation, weighing and storage were performed in laboratories isolated from the animal quarters. Amounts of food sufficient for one week were prepared and stored in tightly covered cans at room temperature. Daily food allotments, manually weighed to the nearest 0.1 gm, were made between 8 and 9 AM each day. Fresh water was given all animals every second day, unless necessary at shorter intervals.

Diets and feeding methods. This longevity study may be divided conveniently into two series: one, to be referred to as the "restricted series," consisted of 5 groups of rats, 4 of which were allotted 4 semisynthetic diets on a restricted basis, whereas rats in the 5th group ("controls") were maintained unrestricted with commercial rations;[2] the other study, which will be referred to as the "unrestricted series," consisted of 4 groups of rats receiving the same 4 experimental diets used in the former series, but allotments were made on an unrestricted basis. The daily allotment to rats and the consumption by them of every dietary component were known throughout life for all diets used in both studies. The purpose of maintaining one group of rats with an unrestricted intake of commercial ration was to permit other workers to compare their data with ours and, in addition, to provide reference points from which to better assess the relative changes exerted by the 8 experimental nutritional regimens. Table 1 shows the composition of each diet.

A. *Restricted series.* The 4 semisynthetic diets were designed to afford different intakes only of casein and of sucrose. This was accomplished by restricting the allotment of each diet by different amounts. The intake of each component in the 4 diets is shown in table 2; these intakes obtained at a time when rats receiving diets A and C were alloted 10 gm of food per day. The precise amounts of food, of calories and of casein at times other than when rats fed diets A and C received 10 gm, have been presented earlier (Ross, '59). Allotments of casein were identical and high in two diets (A and B) and casein allotments were identical and low in the other two. In one of each of these pairs of diets the sucrose allotments were identical and low.

[2] Purina Laboratory Chow, Ralston-Purina Company, St. Louis.

TABLE 1

Semisynthetic diets

Ingredients	Diet A	Diet B	Diet C	Diet D
	%	%	%	%
	Composition			
Casein[1]	30.0	50.85	8.0	21.62
Sucrose	61.0	33.90	83.0	54.05
Corn oil[2]	5.0	8.47	5.0	13.52
Salt mixture (USP 12)	4.0	6.78	4.0	10.81
Vitamins and trace elements[3]				
	Caloric values			
Calories/gm	4.09	4.15	4.09	4.24
Calories supplied by casein, %	29.3	49.0	7.8	20.4

[1] Vitamin-free Test Casein, 89% protein nitrogen, General Biochemicals, Inc., Chagrin Falls, Ohio.

[2] Mazola, Corn Products Refining Company, New York.

[3] Vitamin content of diets adjusted to afford identical allotments each day to each rat of the restricted series: thiamine·HCl, 20 μg; riboflavin, 25 μg; pyridoxine, 20 μg; Ca pantothenate, 0.1 mg; niacin, 0.1 mg; folic acid, 20 μg; vitamin B_{12}, 0.3 μg; menadione, 20 μg; choline chloride, 10 mg; α-tocopherol, 1 mg. Vitamins A and D supplied separately by addition of 5 drops of cod liver oil to food bottle once a week. Trace element content of diets adjusted to afford identical allotments each day to each rat of the restricted series: $MnSO_4 \cdot 4H_2O$, 40 μg; $CuSO_4 \cdot 5H_2O$, 40 μg; $ZnCl_2$, 40 μg; $CoCl_2 \cdot 6H_2O$, 0.4 μg.

TABLE 2

Restricted food allotments of semisynthetic diets[1]

Ingredients	Diet A	Diet B	Diet C	Diet D
		gm/day/rat		
Casein	3.0	3.0	0.8	0.8
Sucrose	6.1	2.0	8.3	2.0
Corn oil	0.5	0.5	0.5	0.5
Salt mixture (USP 12)	0.4	0.4	0.4	0.4
Vitamins and trace elements[2]	—	—	—	—
Total food allotted	10.0	5.9	10.0	3.7

[1] Relative allotments; actual amounts at different chronological ages, 10% below amount consumed by "pilot" rats maintained with diet C ad libitum.
[2] For vitamins and trace elements, see footnote of table 1.

To be sure that the daily food allotment to each rat in the 4 groups would be completely consumed during the longevity study, measurements were made of the intakes of rats given these 4 diets on an unrestricted basis in a pilot study. In this pilot study individual, daily weighed amounts of food (based on previous experience) were offered to these rats in excess of their normal ad libitum consumption and the amount consumed in 24 hours was determined. The rats receiving diet C consumed the least amount of food, and were continued as the "pilot" group for which daily intakes of each of the 25 rats were recorded throughout life. Variations in intakes of individuals, as well as of the group, from day to day were smoothed out by using overlapping, two-week, least square trends. An allotment 10% below each two-week trend was then given to rats receiving diet C and to rats receiving diet A, thus assuring isocaloric intakes of these two groups. Rats fed diet B received 59%, whereas rats supplied with diet D received 37% of this amount. Intakes of rats receiving restricted rations may be characterized as follows: diet A, high casein-high sucrose; diet B, high casein-low sucrose; diet C, low casein-high sucrose; diet D, low casein-low sucrose. Intakes of fat,[3] minerals, vitamins,[4] and trace elements were kept constant, regardless of group.

The number of rats chosen from different litters at random, used in this series, were 210, 210, 120, 210 and 195 for commercial ration, diet A, diet B, diet C and diet D, respectively. Except for those rats chosen at random and sacrificed for biochemical assays, all rats were observed until their death dates. Actuarial tables were calculated (Dublin et al., '49) on a basis of 100-day periods by the use of the number of animals for each group entering that period and by correcting for animals killed during that period. The first period, of necessity, includes the first 21 days of life even though none of the animals were on test, and it is assumed that no deaths occurred. Deaths were classified natural, sacrificial and accidental. The last classification (approximately 1% of the entire population) included only those animals dying as a result of handling mishaps.

B. *Unrestricted series.* The same 4 semisynthetic diets used in the preceding series were given on an unrestricted basis to 4 groups of 25 rats per group. The intakes of these rats, as well as of those maintained with the commercial diet, are shown in table 3. No animals were sacrificed for biochemical assays. The actuarial table was constructed as in the restricted series.

RESULTS

Mortality data obtained from rats fed commercial diet. Mortality distribution is shown in figure 1; the distribution curve is nearly symmetrical. The mean and mode almost coincide at 730 days. Approximately 70% of the rats in this group died between 600 and 900 days of age, the last one dying at 1072 days.

[3] All corn oil used in these experiments was donated by the Corn Products Refining Company, New York.

[4] The author acknowledges the substantial supplies of vitamins donated by Hoffman-LaRoche, Inc.; Lederle Laboratories, Division of American Cyanamid Company; and Merck and Company.

TABLE 3

Unrestricted consumption of commercial and semisynthetic diets

Dietary group	Initial intake			Average increment/day (between ages 21–70 days)	Maximal intake		
	Food	Cal.	Casein		Average food/day	Average Cal./day	Average casein/day
	gm		*gm*	*gm*	*gm*		*gm*
Commercial	6.5	20.4[1]	—	0.79	25.0	78.5	—
A	4.2	17.2	1.26	0.30	17.4	71.2	5.22
B	4.9	20.3	2.49	0.33	18.8	78.1	9.56
C	4.8	19.6	0.38	0.07	15.0	61.4	1.20
D	5.2	22.1	1.12	0.28	19.6	83.2	4.24

[1] Metabolizable energy (3.14 Cal./gm) as estimated by the Ralston-Purina Company.

The survival curve is shown in figure 2 and indicates that the 50% survival time is approximately 760 days.

Complete mortality rates are shown in table 4. When these data are expressed graphically the mortality rate is almost exponential for approximately 600 days but subsequently this accelerating rate decreases.

Life expectancy for this control population is shown in figure 3 and is characterized by an approximately constant, negative slope for the first 700 days.

Mortality data obtained from rats on restricted intakes of semisynthetic diets. Mortality rates of rats maintained with all 4 semisynthetic diets are shown in table 4. Although the mortality rates of the group

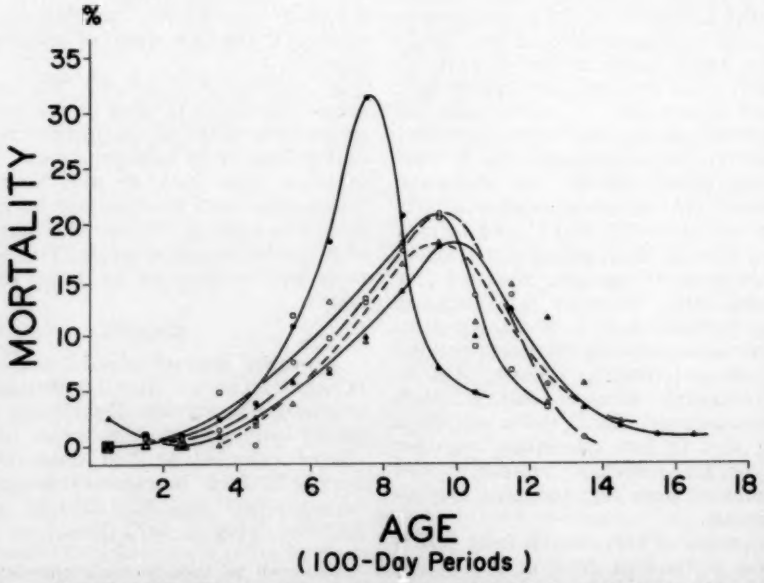

Fig. 1 Mortality in 100-day periods as percentage of each of 4 populations (corrected for rats not dying natural deaths) maintained on restricted intakes of semisynthetic diets, and as percentage of one population (similarly corrected) maintained ad libitum with commercial diet. Key: ●, commercial laboratory chow; ○, high casein-high sucrose intake; △, high casein-low sucrose intake; □, low casein-high sucrose intake; ▲, low casein-low sucrose intake.

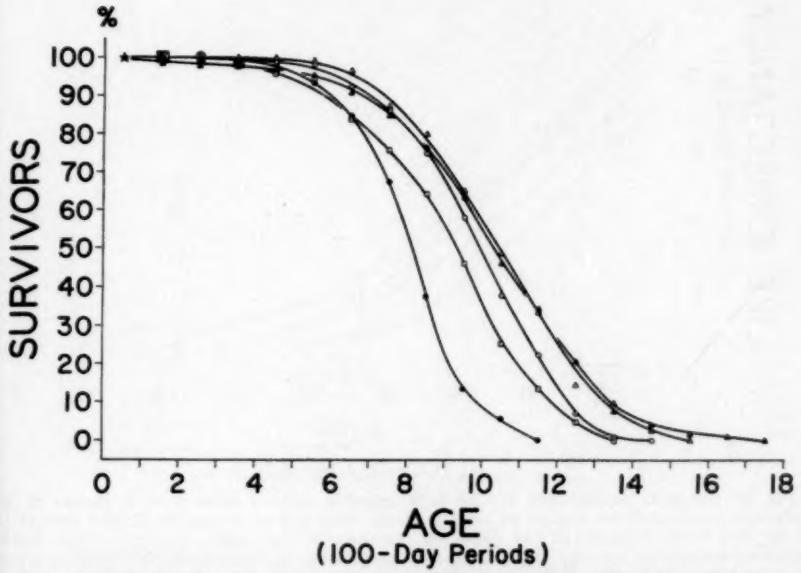

Fig. 2 Percentage of survivors at successive 100-day periods of 4 groups of rats maintained on restricted intakes of semisynthetic diets and of one group of rats maintained ad libitum with a commercial diet. Key: ●, commercial laboratory chow; ○, high casein-high sucrose intake; △, high casein-low sucrose intake; □, low casein-high sucrose intake; ▲, low casein-low sucrose intake.

TABLE 4

Mortality rate (q_x)[1] of rats maintained on restricted intakes of semisynthetic diets

Age period	Dietary group				
	Commercial	A	B	C	D
days	q_x	q_x	q_x	q_x	q_x
21– 99	0	0	0	0	0.016
100– 199	0.005	0	0	0.005	0.006
200– 299	0.005	0.005	0	0	0
300– 399	0.018	0.011	0	0.038	0.006
400– 499	0.034	0	0.012	0.018	0.020
500– 599	0.099	0.063	0.024	0.103	0.048
600– 699	0.201	0.064	0.096	0.100	0.062
700– 799	0.443	0.132	0.079	0.154	0.101
800– 899	0.642	0.224	0.184	0.284	0.174
900– 999	0.600	0.348	0.257	0.447	0.275
1000–1099	1.000	0.408	0.300	0.464	0.245
1100–1199		0.690	0.571	0.667	0.405
1200–1299		0.889	0.333	1.000	0.636
1300–1399		1.000	0.750		0.500
1400–1499			1.000		0.500
1500–1599					0.500
1600–1699					1.000

[1] q_x (mortality rate) is the ratio of the number of rats dying within each 100-day period to the number of live rats entering that period.

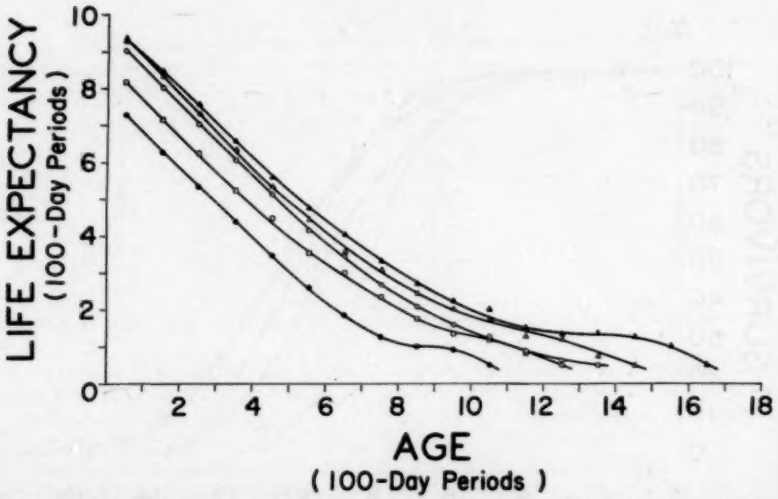

Fig. 3 Complete expectation of life at successive 100-day periods of 4 groups of rats maintained on restricted intakes of semisynthetic diets and of one group of rats maintained ad libitum with commercial diet. Key: ●, commercial laboratory chow; ○, high casein-high sucrose intake; △, high casein-low sucrose intake; □, low casein-high sucrose intake; ▲, low casein-low sucrose intake.

of rats fed diet D during the initial periods were higher than that group of rats supplied with commercial ration, at all later periods mortality rates of rats fed the 4 semisynthetic diets were consistently lower than those of the control group. No deaths occurred during the first 400 days for rats maintained on intakes high in casein and low in sucrose (diet B) and for the next two periods the mortality rates of that group of rats were less than those of the other groups. After the first year of life the group of rats maintained with diets low in casein and high in sucrose (diet C) had the highest mortality rate of the 4 groups. After 900 days the group of rats maintained on low intakes of casein and of sucrose (diet D) had the lowest mortality rate of all.

The changes in mortality rate of each group of rats fed all 5 diets are shown in figure 4. All 5 groups show nonlinearity of the mortality rate-change curve. After 600 days, however, these changes, when expressed graphically as a function of the reciprocal of age, become linear beyond this time period. Hence, the mortality rate may be characterized as an inverse function of the reciprocal of age.

Reduction in the acceleration of mortality (see fig. 4) of the group on low intakes of casein and of sucrose (diet D) is the greatest of all the 5 groups. This is evidenced by its least slope in the later age periods, when compared with earlier periods and also when compared with the other groups. The slopes of the mortality rate curves of the three groups fed diets A, B and C during later periods, are similar to each other and indicate similarity of reduction in the acceleration of mortality.

The beneficial or detrimental influences of the various nutritional regimens upon the rat may be seen also in mortality ratios. Values of mortality ratios for each of the 4 experimental groups are given at two time periods, before and after 700 days of age (table 5). A value of this ratio less than unity (or less than 100 when expressed as per cent) indicates the beneficial effect of the experimental diet over that of the control in terms of mortality expectation, and in each experimental group such an effect was obtained. The influence of diet D in early life on mortality rate of rats is not as beneficial as is that of diet B.

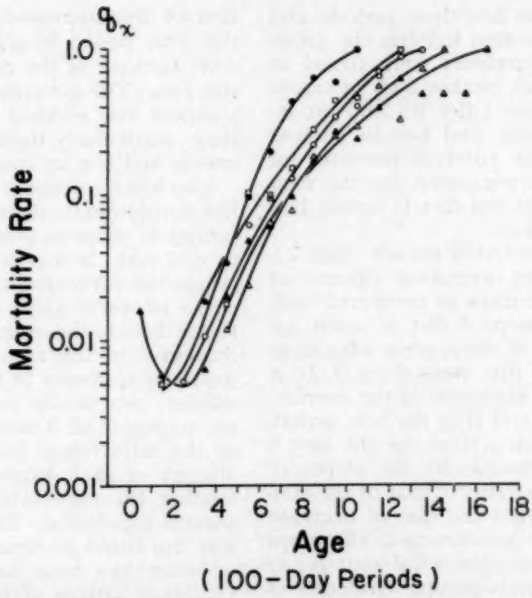

Fig. 4 Complete mortality rates at successive ages of 4 groups of rats maintained on restricted intakes of semisynthetic diets and of one group of rats maintained ad libitum with commercial diet. Mortality rates calculated on the basis of 100-day periods using the number of rats dying within a period against the number of rats alive in the total population of each group entering that period (corrected for rats sacrificed during that period). Key: ●, commercial laboratory chow; ○, high casein-high sucrose intake; △, high casein-low sucrose intake; □, low casein-high sucrose intake; ▲, low casein-low sucrose intake.

TABLE 5

Mortality ratio[1] of rats maintained on restricted intakes of semisynthetic diets

Age periods	Dietary group			
	A	B	C	D
	%	%	%	%
First 700 days	48	35	75	46
After 700 days	34	23	44	22

[1] Mortality ratio (expressed as per cent) computed as number of actual against expected deaths. Expected death rate equals mortality rate at each time period of control population × exposure of experimental population.

The beneficial effect of the semisynthetic diets as contrasted with the effect of the commercial diet upon mortality, is seen in the mortality distribution patterns shown in figure 1. The mode value of each group approaches closely the values of the average life span, which for rats on diets A, B, C and D are, respectively, 904, 935, 818 and 929 days. The longest-lived rat in each group died at 1322, 1480, 1287 and 1638 days on diets A, B, C and D, respectively.

The percentage survival curve (fig. 2) indicates a population half-life of 940, 990, 880 and 990 days for rats on diets A, B, C and D, respectively. The percentage of survivors to successive age periods for the different groups of rats remained approximately the same for the first three age periods although deaths in the early part of the first period for rats in group D separate this group from the others in early periods.

Rats fed the commercial diet and rats with intakes low in casein and high in sucrose (diet C) have similar survival values for the next three periods. After this time, however, the lesser slope of this curve indicates the beneficial effect of this diet over the control diet. Rats fed diets A, B and D had a consistently higher sur-

vival rate after the first three periods, and for the first population half-life the greatest number of survivors were found in groups of rats with intakes high in casein and low in sucrose (diet B) and on intakes low in casein and low in sucrose (diet D). Similar survival patterns for these two groups were seen for the next 4 periods, but rats fed diet D finally had the greatest survival.

When life expectation curves, (fig. 3), are examined, the increased efficacy of the semisynthetic diets as compared with that of the commercial diet is again apparent. In order of decreasing advantage to expectation of life, were diets D, B, A and C. With the execption of the overlapping of curves B and D in the first period, there is a decreasing slope for the first 6 or 7 periods; subsequently the slopes of all 4 groups are parallel to that of the controls, indicating that the rate of decrease in life expectation is common to all groups even though expectation is different at any one period for each group. Evidence of the relative extension of life expectancy by use of the various semisynthetic diets is also seen in the differences in time at which the parallelism of the curves begins to change and at the time when life expectancy approaches zero.

The improvements in life expectancy of the experimental populations over the controls up to and including the 10th period are shown in table 6. No indication of the increased advantage beyond the 10th period is given for the various diets because of the death of all the control rats. The actuarial table accents the prospect for survival of rats after this time, particularly those on intakes low in casein and low in sucrose.

TABLE 6

Life expectation of rats maintained on restricted intakes of semisynthetic diets

Age period	Increase in life expectancy[1]			
	Dietary group			
	A	B	C	D
days	%	%	%	%
21–99	24	28	12	27
100–199	28	33	14	35
200–299	32	38	17	42
300–399	39	45	19	50
400–499	49	54	29	62
500–599	61	71	37	84
600–699	86	94	61	117
700–799	113	145	86	163
800–899	108	142	75	170
900–999	78	122	48	148
1000–1099	160	250	142	302

[1] Computed as percentage increase with respect to life expectation of rats consuming commercial rations.

The life expectancy curves for the rats fed semisynthetic diets have marked similarities in shape to that of the curve of the control rats. It was possible, therefore, to adjust the ordinates and abscissae of the curve of each group receiving semisynthetic diets to the ordinate and abscissa of the curve of the control group. The adjustment is shown in figure 5 and the resulting, practically indentical cluster of the points of all 5 curves suggests the use of the adjustment factors as indexes of efficacy of each experimental diet in extending life expectation over that of the control population. These adjustment factors are listed in figure 5, and the value of unity has been assigned the life expectation pattern of the control group as a point of reference.

Mortality data of rats on unrestricted intakes of semisynthetic rations. The life span data of rats consuming the same 4 semisynthetic diets on an unrestricted basis were, with the exception of one group (diet C), markedly different and of shorter duration than those observed when rats were maintained with these diets on a restricted basis.

The average life span of rats supplied with diets A, B, C and D are, respectively, 305, 596, 832 and 600 days. The 50% survival times of rats fed these same diets are 305, 680, 910 and 650 days, respectively. The longest-lived rats in the group died at 347, 810, 1251 and 895 days, receiving diets A, B, C and D, respectively, and the average life-time mortality ratios, expressed as per cent, for these same groups are 5000, 246, 56 and 210, respectively.

The mortality rates and life expectancies of this series are given in table 7. The shortness of life of rats consuming diet A has been confirmed by repeated studies. The average life spans for rats supplied with diets B and D are almost identical and, although mortality rates are

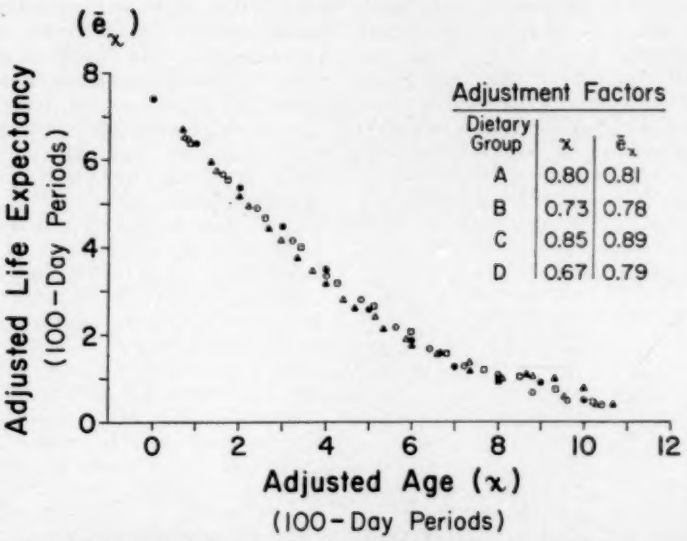

Fig. 5 Patterns of adjusted life expectancy-adjusted age period. Life expectancy curves of 4 groups of rats maintained with semisynthetic diets (see fig. 4) were adjusted to that of the curve of rats maintained with commercial diet by using two adjustment factors (see also fig. 4). The factors for each curve were obtained by dividing the value of the life expectancy of each of the 4 experimental populations during the first period, into the value of the life expectancy (ordinate) of the control population for the same period; similarly, the value of the last age period of each of the experimental populations was divided into the value of the last age period (abscissa) of the control population. All data of life expectancy and of age periods for each of the 4 experimental groups were multiplied by these two quotients in order to make final adjustment. Values for the control population are identical with those in figure 3. Key: ●, commercial laboratory chow; ○, high casein-high sucrose intake; △, high casein-low sucrose intake; □, low casein-high sucrose intake; ▲, low casein-low sucrose intake.

TABLE 7

Mortality rate (q_x)[1] and life expectancy (e_x)[2] of rats maintained on unrestricted intakes of semisynthetic diets

Age period	Mortality rate				Life expectancy in days			
	Dietary group				Dietary group			
	A	B	C	D	A	B	C	D
days	q_x	q_x	q_x	q_x	e_x	e_x	e_x	e_x
21–99	0.076	0	0	0	288	583	830	598
100–199	0.042	0.089	0	0	208	483	730	498
200–299	0.348	0	0	0	115	423	630	398
300–399	1.000	0.136	0.080	0.200	50	323	530	298
400–499		0	0	0.200		266	472	260
500–599		0.263	0.087	0.125		166	372	213
600–699		0.500	0.095	0.429		107	302	136
700–799		0.857	0.158	0.500		64	229	100
800–899		1.000	0.375	1.000		50	163	50
900–999			0.500				130	
1000–1099			0.600				110	
1100–1199			0.500				100	
1200–1299			1.000				50	

[1] See footnote 1, table 4.

[2] e_x is the average future lifetime of rats at age period indicated.

different in nearly all periods, the small number of rats in each group precludes any analysis for significance. The rats supplied with diet C are the only group which had a mortality ratio less than unity, thus indicating the beneficial effect of this diet on an unrestricted basis as compared with that of the control diet.

An adjustment factor for the life expectation slopes cannot be presented with sufficient accuracy because of the small number of rats involved.

DISCUSSION

The experimental extension of life span of rats by the restriction of intake of total food or of calories has been correlated with retardation of growth for limited periods of time, notably by McCay and his associates ('52), although others have questioned this relationship. In the more recent studies on mortality by Berg and Simms ('60), who used two levels of restriction of a commercial diet over an 800-day period, a trend toward lower mortality rates was indicated. Except for isolated reports which have been reviewed earlier (Ross, '59, '60), little direct information is available concerning the long range consequences of nutritional imbalance such as overnutrition or undernutrition of basic food elements. Furthermore, the unique but ever-changing requirements of age rarely have been considered.

In the studies presented here several fundamental differences from those of other workers obtain: (a) a single nutritional state, both qualitative and quantitative, was maintained for life; (b) evaluation was made of two levels of protein (two groups each) with varying levels of carbohydrate and, also, of one level of carbohydrate (two groups) with varying levels of protein; (c) identical daily intakes of fat, minerals, vitamins and trace elements were assured for each rat in the restricted series throughout life; and (d) a comparison was made between unrestricted and restricted feeding of all experimental diets.

From the data presented, life span is definitely a function of the lifelong dietary regimen. In general, the semisynthetic diets result in longer life spans when fed on a restricted basis than when fed on an unrestricted basis. The only exception to this observation is the diet low in protein and high in carbohydrate (diet C) which, when fed ad libitum, is equally as effective in enhancing life span as when fed on a restricted basis. This exception may be the result of the relative proportions of the ingredients in the diet or of the relatively small level of restriction imposed, or both. Unfortunately, there are no published data which indicate either the maximal level of restriction required for optimal benefits to life span, or which indicate the minimal level of restriction which just begins to enhance life span. The relationship between beneficial effects and degree of caloric restriction was indicated by Tannenbaum and Silverstone ('53) who correlated the incidence of tumors (as probits) in the mouse, to the logarithm of the caloric intake.

When administered on an unrestricted basis, a diet low in protein and high in carbohydrate (diet C) is shown to be superior to those diets with higher levels of protein or those with lower levels of carbohydrate by all actuarial techniques used. When these latter diets are given on a restricted basis, however, this superiority of diet C on an unrestricted basis is lost, since even longer life spans are produced by the other diets. Under unrestricted conditions, however, the total intake of food is higher for groups of rats on diets A, B and D than for the group on diet C. Hence, this self-imposed limitation in food consumption, or reduced desire for food, which is generally seen in rats fed diets with levels low in protein and high in carbohydrate, may account for the relatively longer life span. Probably related to this phenomenon of reduced desire for food are the small but consistent differences in mortality of rats maintained upon a restricted basis with diets A and C. Since these diets were isocaloric in value and in intake, there must have been a considerably greater restriction for those rats with the greater desire for food (diet A). In a similar way, life spans were enhanced when externally imposed restrictions upon mice with a genetic propensity for overeating (obese), limited their weight to

the "normal" (nonobese), when compared with both the control obese mice with ad libitum intakes and with the "normal" nonobese mice. (Lane and Dickie, '58).

In the unrestricted series of our study, small differences in intake of calories or of total food cannot explain the remarkable differences in life span between those rats maintained with diet A and those supplied with diet B or D. Since dietary composition influences appetite, the relative degree of "internal restriction" must be taken into account in evaluating dietary influence upon life span. Greater restriction in total intake of diet C might have resulted in longer-lived rats but the design of this study, in which similar intakes of protein were essential, would not allow further restriction in the allotment of this diet because of the severe limitation that would be imposed upon the rats allotted diet D.

Although growth data are usually considered in the presentation of experimental studies of life span, in this study there are multiple correlations of growth with the other parameters mentioned above and investigated in the course of the larger, longevity study. Hence, growth alone cannot be considered in this paper and will be discussed in a later communication. In view of McCay's correlation between retardation in growth and length of life, however, it might appear that there would be a relationship also between maximum weight attained by each group and the length of life. Although this relationship obtains for rats on restricted intakes, (maximum weight being 440, 282, 394 and 167 gm for dietary groups A, B, C and D respectively), it does not for those consuming food on an unrestricted basis, (maximum weights being 610, 366, 606, 455 and 610 for dietary groups, commercial diet A, B, C and D, respectively). Relationships between nutritional states and growth patterns, namely, efficiency of food utilization, do have some bearing on life span. In our experience[5] those groups of rats on restricted intakes with extended life spans require at least 2.5 times more protein to double their weight at 50 gm than is required for the other, shorter-lived groups, including those receiving commercial rations.

In evaluating the effect of nutritional regimens upon life span the value for average length of life affords less information than other aspects of mortality data do. For instance, the values for the average life span of rats maintained on restricted intakes of the 4 semisynthetic diets are not strikingly different, but the difference in patterns of the mortality rate curve is distinctive. This divergence in mortality rate pattern has its counterpart in every analysis made, including life expectancy, percentage of survivors, change in acceleration of mortality and in the mortality ratios.

In early life a particular diet may be found satisfactory for development and maintenance of the organism, but in later life this diet may be unsatisfactory in respect to the development of disease and its effect upon the length of life. Similarly, a diet which may not be conducive in early life to maximal growth potential may, on continued feeding, lead to the lengthening of life span. This is evident when comparing the effects of the same diet fed on an unrestricted or on a restricted basis, and when comparing the effects of two different diets. For example, the average life span of rats whose restricted intake was high in protein and in carbohydrate (diet A) was similar to that of rats whose intake was low in protein and in carbohydrate (diet D). As early as the first year, however, there was noted the beginning of a consistent trend wherein the mortality rate of those rats fed the latter diet decreased, with a resultant increase in number of survivors and, as time progressed, the difference between these two groups was dramatic. Thus life spans for individual rats, far beyond any so far recorded, have been obtained when use was made of a protein nitrogen allotment of slightly more than one gram per kilogram of body weight and a reduced intake of carbohydrates and of calories.

The differences in changes of mortality rate between the various populations in

[5] Ross, M. H., unpublished data.

the restricted series were most evident in late life. During this phase of life the mortality rate curves of each of these populations approached linearity when plotted as a function of the reciprocal of the age. The slopes of these lines for rats supplied with diets A, B and C are nearly identical and greater in value than that found for rats maintained with diet D. Thus, this last population with the relatively lesser slope during the log phase, is further favored during the terminal phase. Even though a few rats died early in life in this group, the diminishing slope in late life suggests that the nutritional requirements are being met more suitably at these ages than heretofore. Common environmental conditions, including time and random genetic constitution, preclude the argument that selective deaths early in life influenced the population-age characteristics later in life.

Although no reference has been made to the state of health throughout life, including incidence and type of organic disease, those physiologic and pathologic processes that are inimical to life unquestionably have been retarded for the rats consuming diet D (Ross, '59). Had it not been for formation of trichobezoars, the cause of 45% of deaths during the third and fourth year of life, it is reasonable to suppose that life spans of rats in this group would have been extended even further.

The differences in caloric intake between pairs of groups of rats of the restricted series maintained on identical amounts of protein (diets A and B, and diets C and D) make it difficult to assess the influence on longevity of the precise amounts of protein consumed. In both cases where the intake of protein was identical, high or low, the one group of each pair restricted in carbohydrate intake and therefore in caloric intake, had longer life spans, and the longest-lived rats were obtained when the protein and the carbohydrate intake was the lowest. Although that group allotted the higher level of protein sustained no deaths for some 500 days, this was not the case for those rats allotted low intakes of protein. These deaths early in life for rats fed diet D, while not susceptible to statistical treatment, nevertheless lead one to suspect that they were directly related to the stress of the severe dietary deprivation in the first few weeks of study (continuous loss in weight, and intestinal impaction as the common cause of death). Even with this adverse beginning, beneficial influences of continued feeding of this diet became evident after some 700 days and continued in evidence until some 160 days after the last rat had died in all other groups.

When these same semisynthetic diets were fed on an unrestricted basis the spectrum of life span patterns was quite different from that obtained when they were fed on a restricted basis. Of particular interest was the unexpected, sudden demise of the population of rats maintained with diet A, since total food consumption levels and weight-gain patterns of these rats were not greatly different from those receiving diets B and D. Furthermore, there was no apparent relationship between length of life and the protein or caloric intake, in that both these intakes were found to be intermediate to those of rats fed diets B and D. Under conditions of restriction, whether spontaneous or administered, enhancement of life span may be accomplished by the reduction in intakes of total dietary components, whereas under unrestricted conditions the relationship of the dietary constituents to each other assume a larger role.

In earlier short-term studies (Ross '54a, b, '56, '57) it was shown that certain quantitative relationships existed among hepatic enzyme activity patterns, protein-carbohydrate relationships, and aging. It was possible by modification of the proportion of the protein and carbohydrate components in the diet of rats fed on an unrestricted basis, to alter the activity patterns which were characteristic of the age and the diet, to patterns characteristic of other chronological ages. In correlating these observations with those made on length of life of rats maintained on an unrestricted basis, it was found that a direct relationship existed between the hepatic enzyme activity response to the diet

and the average length of life of the rat. Those rats maintained with diet A had an enzymatic response similar to that observed in older rats and the shortest average life span, whereas rats maintained with diet C had an enzymatic activity pattern more like that of the young rat and had the longest average life span. For rats supplied with diets B and D, which contained 22% and 51% protein, respectively, the average life spans were the same, and the enzyme activity responses of rats maintained on such diets were not only similar to each other but were intermediate to those obtained in rats fed A and C.

Uniformity in life expectancy patterns among the different groups of rats on restricted intakes suggests that under the conditions of this study and for this strain of rats, there may be a basic mortality pattern which differs only in its rate of development. Had these studies on the rats with restricted intakes been terminated at 500 days, it would have been difficult to extrapolate earlier observations in order to estimate the full effect of diet on length of life. The pattern noted after 700 days leaves no doubt as to the beneficial influences of restriction. Whether this delay in change in mortality trend is the result of the cumulative nutritional status prior to this age, or whether the animal must reach a specific age before the nutritional state exerts its dominant effect remains to be determined.

The potential life expectancy of each population can be considered to have been identical at the time of initiating this program, but the potential was not as fully expressed in the shorter-lived rats as in the longer-lived ones. The phrase "extension of life span" is generally considered to mean an increase in the length of life beyond the average demographic "norm." The upper limit of the potential life span of these rats, however, is not yet known. Rather it can be assumed that it may be considerably longer than any so far observed, especially when the organism is presented with optimal environmental conditions in addition to optimal nutritional conditions. For this reason, it may be erroneous to state that rats with the longest life span are those that have had their length of life extended. The longer-lived populations may be approaching their potential expectancy, whereas the shorter-lived ones in actuality have had their life expectancy decreased by the effects of the diet.

In these studies only one nutritional scheme was used for each population. Furthermore, each population has been limited to the influences of quantitative variation in one protein, one carbohydrate and in calories; but the role of fat, minerals, vitamins and other essential food substances, as well as of the biological value and physical properties of the total food, must be considered in this complex relationship.

The question of "adequacy" must be re-examined in view of the possibility that nutritional requirements as they change with advancing age, may make it necessary not only to continuously alter dietary components quantitatively but also to alter them qualitatively in order to attain maximal extension of life expectancy.

SUMMARY

The effect of uniform lifelong dietary regimens on the mortality patterns of rats has been investigated. More than 1000 male rats divided into 8 groups received one of 4 experimental diets per group which were allotted on a restricted as well as on an unrestricted basis. The various dietary regimens were begun at weaning age. The influence upon mortality patterns of precise intakes of protein (casein) and of carbohydrate (sucrose) and of variation in the ratios between these two components in an otherwise "adequate" diet, has been assessed.

Actuarial tables have been constructed for each of the populations and include determination of mortality distribution, mortality rates, survival pattern and life expectancy at successive ages. Mortality ratios and life expectancy indexes of the populations consuming semisynthetic diets have been computed.

The effects obtained by restriction of intake of the experimental diets were, in general, more beneficial for all length-of-life parameters measured than when the

diets were administered on an unrestricted basis. Restricting the intake of the protein component only, in two diets allotted on an isocaloric basis had little effect. Restricting the intake of carbohydrate only with simultaneous restriction of caloric intake enhanced life expectancy. Restricting the intake of both protein and carbohydrate with simultaneous restriction of caloric intake, though showing no pronounced effects early in life, enhanced life expectancy to the greatest degree because of the beneficial effects later in life.

When these same diets were fed on an unrestricted basis there were greater differences in mortality patterns between each population than were found when feeding was restricted. Under unrestricted conditions a relationship between length of life and intake of protein, carbohydrate, and the proportion of these components of the diet, was found for only one group. Rats in this group which received a diet low in protein and high in carbohydrate restricted their own intake and therefore their total intake of calories and had the longest life span.

Length of life has been found to be influenced not only by quantitative dietary restrictions but also by the ratio of the protein and carbohydrate components in the diets.

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Effects of Zinc Deficiency in the Diets of Hens[1]

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Several reports have appeared in recent years concerning the effects of zinc deficiency in the diets of young chicks and poults. Few reports have considered the effects of zinc deficiency in diets of mature birds. Supplee et al. ('58) reported that zinc, added to a diet containing isolated soybean as the protein component, improved hatchability of fertile eggs and slightly increased shell thickness. Edwards et al. ('59) observed that chicks produced by hens fed a zinc-deficient diet grew more slowly than normal. Turk et al. ('59) reported that hatchability was decreased when hens were fed a soy protein diet of low-zinc content (8 to 9 ppm) and that the chicks hatched were weak, poorly feathered, and usually died within a few days. Blamberg et al. ('60) reported that, in addition to the previously described deficiency symptoms, impaired embryonic development resulted from zinc-deficient maternal diets.

In view of the lack of information on the effects of zinc deficiency on hens and their progeny, and our inadequate knowledge of the zinc requirement of hens, two experiments were conducted. The first experiment (1958–59) was designed to test the difference between casein and isolated soybean protein in a low-zinc diet, and the effects of dietary calcium level on zinc deficiency. The second experiment (1959–60) was designed to study the nature of the embryo abnormalities produced by the low-zinc, soy protein diet.

EXPERIMENT

Experiment 1. Thirty Single Comb White Leghorn hens were divided into 6 groups of 5 birds each and placed in a plastic-coated hen battery. Distilled water was provided in a plastic and stainless steel watering system. Feed was provided ad libitum in aluminum feeders. The diets used are shown in table 1. Lots 1 and 2 received the casein-sucrose diet, adapted from the chick diets of Morrison et al. ('56) but containing 4.0% of calcium. This basal diet (lot 1) contained 15 ppm of zinc, by analysis, for the first 26 weeks of the experiment, but after a change in casein source the zinc content decreased to 10 ppm for the remainder of the experiment. Lot 2 received approximately 55 ppm of additional zinc as zinc car-

TABLE 1
Basal diets

Ingredient	Casein	Soy protein[5]
	gm/kg	*gm/kg*
Sucrose	604.3	630.3
Crude casein	182.0	—
Soy protein[1]	—	200.0
Cellulose[2]	30.0	30.0
Choice white grease	50.0	50.0
Vitamin mix[3]	2.5	2.5
Choline chloride (70%)	2.2	2.2
α-Tocopheryl acetate	0.025	0.025
Mineral mix[4]	70.0	70.0
$CaCO_3$	59.0	15.0

[1] ADM C-1 Assay Protein, Archer-Daniel-Midland Company, Cincinnati.

[2] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[3] Provides in mg/kg: thiamine·HCl, 10; riboflavin, 10; niacin, 50; Ca pantothenate, 20; pyridoxine·HCl, 4.5; biotin, 0.2; folic acid, 4; menadione, 0.5; vitamin B_{12}, 2 μg; vitamin A, 7500 IU; vitamin D_3, 1500 ICU.

[4] Provides/kg: (in grams) $CaHPO_4$, 21.51; $CaCO_3$, 28.2; K_2HPO_4, 11.12; NaCl, 6; $MgSO_4$, 2.5; and (in milligrams) ferric citrate, 333; $MnSO_4$, 333; KI, 2.6; $CuSO_4 \cdot 5H_2O$, 16.7.

[5] The high-calcium, soy protein diet contained 59 gm of calcium carbonate and 586.3 gm of sucrose in place of the values shown here.

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bonate. The other 4 groups were fed the isolated soybean protein[3]-sucrose diets that were also adapted from those of Morrison. The normal-calcium, soy protein basal diet (lot 3) contained 2.25% of calcium and 10 ppm of zinc and the high-calcium, soy protein diet, 4.0% of calcium (lot 4). These two diets were also fed with 55 ppm of supplemental zinc as the zinc carbonate (lots 5 and 6). After 26 weeks the zinc content of the diet fed lot 4 was increased to 18 ppm (by analysis) by the addition of $ZnCO_3$. The zinc content reported for each diet represents an average obtained by the analysis of each batch of feed. Distilled water, used as drinking water, contained an insignificant amount of zinc.

All hens were artificially inseminated at weekly intervals with pooled semen from New Hampshire males fed commercial-type diets. Eggs were collected daily and incubated weekly. All eggs were identified as to dam and all chicks that hatched were identified as to dam with a wing band. Some of the resulting chicks were used in the zinc-injection trials. All live chicks were placed in galvanized electrically heated batteries after being wing banded, and were fed a practical-type chick starter ration and tap water. Chicks from hens fed normal and high-calcium, soy protein basal diets were given intraperitoneal injections of a 1.4% solution of zinc chloride in water, or were given an oral dose of the same solution. Dilutions of the zinc solution were made with 0.9% zinc-free saline solution to keep the injection solution isotonic. The normal chicks used to establish zinc tolerance were either chicks from the zinc-supplemented experimental lots or chicks produced by similarly mated hens that had been fed practical-type rations. There was no difference in tolerance to zinc injection or to oral zinc due to chick source.

Two and 32 weeks after initiation of the experiment, 6 eggs from each lot were separated into yolk and white and then frozen for zinc analysis. Within each treatment, the 6 yolks were pooled and mixed prior to freezing. Whites were handled similarly. Thickness of 12 to 18 egg shells from each treatment was determined at the same time.

After 38 weeks on experiment, the hens were killed and tissue samples removed and frozen for analysis. All zinc analyses were made according to the method of Vallee and Gibson ('48) as modified by Hoch and Vallee ('49). Blood samples were drawn by heart puncture into a zinc-free citrated syringe. Each sample was centrifuged and the plasma and red cells were separated by decantation. Separated red cells and plasma were then frozen. These samples were thawed just before analysis and 10 ml of plasma or 2 gm of red cells were ashed at 700°C for 16 hours in a platinum crucible. The ash was then taken up in 6 N HCl and the method of Hoch and Vallee followed from this point.

Experiment 2. This experiment was conducted the following year in the same environment. The hens used were from the same line of stock. Diets 3 and 4 were used with and without 55 ppm of supplemental zinc. Dithizone method of zinc assay showed the diet to contain 10 ppm of zinc, and 68 to 74 ppm (air-dry basis) after addition of $ZnCO_3$. Egg production, embryo, and chick growth were studied. Photographs and color slides were made of abnormal embryos.

RESULTS AND DISCUSSION

Experiment 1. Hens receiving the soy protein-type diet failed to gain normally when fed lower levels of either calcium or zinc, but gained significantly ($P < 0.01$) more when fed the higher levels of calcium and zinc simultaneously. Hens receiving 4% of calcium in a casein-type diet gained the same regardless of zinc level in the diet. It is apparent that the isolated soybean protein introduces a complicating factor. This product interferes with the utilization of zinc (Kratzer et al., '59), and it may interfere with the utilization of calcium (table 2, lots 5 and 6). Egg production (table 3) was quite markedly decreased in the low-zinc groups. The addition of extra calcium to the soy protein diets appeared to depress the egg production irrespective of zinc level. No significant differences of shell thickness

[3] ADM C-1 Assay Protein, Archer-Daniel-Midland, Cincinnati.

TABLE 2

Gain in weight of hens as affected by interrelationships of dietary zinc, calcium and protein source (exp. 1)

Lot	Dietary treatment: Protein source	Ca	Zn	Hen weight: Initial weight	After 26 weeks	Gain[1] 0–26 weeks
		%	ppm	gm	gm	gm
1	Casein	4.00	15	1783	2060	277 ab $\alpha\beta$
2	Casein	4.00	70	1680	1930	250 bc $\alpha\beta$
3	Soy protein[2]	2.25	10	1585	1660	75 c β
4	Soy protein	4.00	10	1554	1598	44 c β
5	Soy protein	2.25	65	1795	1815	20 c β
6	Soy protein	4.00	65	1722	2180	458 a α

[1] Latin letters in column refer to significance at the 5% level and Greek letters to significance at the 1% level. Treatments with a common letter are not significantly different at the level indicated. Treatments without a common letter are significantly different at the levels indicated.

[2] ADM C-1 Assay Protein.

TABLE 3

Effect of type of diet and zinc level on egg production and shell thickness (exp. 1)

Lot	Diet	Av. production hen-day 0–38 weeks	Av. shell thickness at 32nd week (10^{-3} in.)
		%	
1	Casein, 4.0% Ca, 15 ppm Zn[1] (10 ppm after 26th week)	46.3	11.0[2]
2	Casein, 4.0% Ca, 70 ppm Zn (65 ppm after 26th week)	60.0	11.2
3	Soy protein,[3] 2.25% Ca, 10 ppm Zn	49.5	10.7
4	Soy protein, 4.0% Ca, 10 ppm Zn 0–26th week. 18 ppm Zn 26–38th week	36.5	12.0
5	Soy protein, 2.25% Ca, 65 ppm Zn	57.5	11.4
6	Soy protein, 4.0% Ca, 65 ppm Zn	52.0	12.1

[1] By analysis; air-dry basis (feed containing 2% H_2O).

[2] No significant ($P < 0.05$) differences among treatments.

[3] ADM C-1 Assay Protein.

resulted from variation of either zinc or calcium in the diet. The number of egg yolks analyzed for zinc content was too few to allow definite conclusions, but there was some indication that zinc concentration was lowered in the yolks by withholding zinc from the maternal diet containing soy protein. The amount of zinc in the white of the egg was so low that it could not be accurately measured by the method of analysis used.

Results of hen blood analyses for zinc and hemoglobin are shown in table 4. No difference due to treatment was observed in the amount of zinc in red cells. Plasma levels of zinc varied more between groups, and more between individuals within a group, than did the red cell zinc. The zinc concentration in the plasma appeared to decrease with the addition of excess calcium to the diet, but concentration of plasma zinc appeared little affected by dietary zinc level.

An increase in hemoglobin levels in chicks suffering from zinc deficiency has been reported by Norris and Ziegler ('58), who found it to be a normocytic polycythemia. In the present study with hens, no significant hemoglobin difference was observed (table 4). Furthermore, our

TABLE 4

Effect of supplemental dietary zinc on zinc and hemoglobin content of blood from hens (exp. 1)[1]

Diet	No. birds	Plasma zinc	Red cell zinc	Hemoglobin
		ppm	*ppm*	*gm/100 ml*
Soy protein,[2] 2.25 % Ca, 10 ppm Zn	4	4.78[3]	12.3[3]	9.6[3]
Soy protein, 4.0% Ca, 10 ppm Zn 0–26 weeks, 18 ppm Zn after 26 weeks	5	2.95	12.2	8.2
Soy protein, 2.25% Ca, 65 ppm Zn	4	4.34	11.8	7.4
Soy protein, 4.0% Ca, 65 ppm Zn	5	3.30	12.5	8.1
Difference needed for significance ($P < 0.05$)		1.91	1.67	2.6

[1] All measurements made at 38 weeks. Data are average of surviving hens.
[2] ADM C-1 Assay Protein.
[3] No significant ($P < 0.05$) differences among treatments.

data show a subnormal hemoglobin level in newly hatched zinc-deficient chicks (table 5).

Results of the hatchability studies are shown in figure 1. For clarity, results from only three of the soy protein diets are shown. The normal-calcium, soy protein diet with added zinc and both of the casein diets gave hatchability results indistinguishable from those observed for birds fed the zinc-supplemented, high-calcium, soy protein diet. Zinc deficiency in the two soy protein diets markedly reduced hatchability after a 6-week depletion period. With the high-calcium, soy protein diet, hatchability soon dropped nearly to zero and remained there until the 26th week, when 10 ppm of zinc was added to the diet. Within two weeks hatchability returned to the control level. Hatchability decreased much less in the lot of zinc-deficient hens fed a normal calcium level. The addition of 55 ppm of zinc to either the high-calcium or to the normal-calcium, soy protein diets maintained normal hatchability, which was also true for the hens fed casein-containing diets, with or without supplemental zinc. As pointed out, the source of casein used for the first 26 weeks contained more zinc than the iso-

TABLE 5

Hemoglobin levels in the blood of day-old chicks hatched from hens fed zinc-supplemented and unsupplemented diets

Maternal diet	Hemoglobin in 100 ml of blood
	gm
Casein, 4.0 Ca, 15 ppm Zn	7.5[1]
Casein, 4.0% Ca, 70 ppm Zn	7.6
Soy protein,[2] 2.25% Ca, 10 ppm Zn	8.8*[3]
Soy protein, 4.0% Ca, 10 ppm Zn	6.6*
Soy protein, 2.25% Ca, 65 ppm Zn	8.1
Soy protein, 4.0% Ca, 65 ppm Zn	8.0

[1] Average of individual determinations on 6 chicks in each lot, from eggs laid the 23rd and 24th week of the experiment.
[2] ADM C-1 Assay Protein.
[3] Starred numbers are significantly ($P < 0.05$) different.

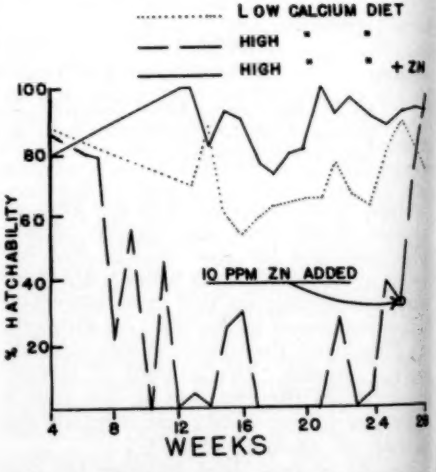

Fig. 1 Experiment 1. Effect of calcium level in zinc-deficient hen diets on hatchability of the eggs. Percentage of hatchability is plotted against the time interval.

lated soy protein; in fact, the casein-containing diets used in the early part of the experiment supplied nearly as much zinc as was curative in the high-calcium, soy protein diet. Therefore, it could not be stated from this experiment whether the zinc requirement of hens fed casein differed from that of hens fed the purified soybean protein.

All eggs that failed to hatch were opened and the embryos examined. No characteristic time peaks of mortality were observed. Most of the embryos that failed to hatch appeared normal on gross examination, but in the high-calcium, soy protein lot, about 5% of the eggs set produced embryos that after 21 days of incubation had completely formed heads and necks with complete viscera attached. However, there was often no skeleton except for skull and some vertebrae; no body wall, wings, legs or feet. Lungs, liver, heart, intestines, and all other internal organs were present and fully developed. In both low-zinc, soy protein groups, a large water blister-like structure formed on the neck and thorax anterior to the wing attachment on some of the dead embryos. Such structures were seen in 2 to 3% of the embryos that failed to hatch and in occasional chicks that hatched. None of these abnormalities were seen in any group receiving supplemental zinc or casein-containing diets. These abnormalities did not appear in the high-calcium, soy protein groups after the addition of 10 ppm of zinc to the ration of the hens. Other random abnormalities were more common in the low-zinc groups, particularly in the high-calcium, soy protein lot, but these same abnormalities occasionally occurred in the zinc-supplemented and the casein-fed groups as well.

Zinc-deficient chicks began to appear in the hatch of the 13th week of the first experiment. These chicks were very weak, and not able to stand. Their breathing rate was much more rapid than normal and was labored. When these chicks were disturbed, the respiratory symptoms became more severe, and in many cases death resulted immediately. These chicks would not eat or drink, and seldom lived more than 3 to 4 days if placed in a starting battery with feed and water and left undisturbed. Feather development at hatching was retarded. In the high-calcium, soy protein group, such chicks were observed only for a short period because later only an occasional chick hatched and the embryo abnormalities described above appeared. In the normal-calcium, soy protein group, deficient chicks continued to hatch regularly, but showed considerable variation in the severity of symptoms within any one hatch and large variations in the relative weakness of the chicks from hatch to hatch. There was no difference in the initial weights of the chicks due to treatments.

To attempt zinc therapy on the zinc-deficient chicks, the tolerance of the chick for zinc salts given by injection or by instillation into the crop was determined. Because the deficient chicks would not eat or drink, these two routes of administration of supplementary zinc could not be used. The results of the zinc tolerance studies using normal chicks are shown in table 6. Chicks tolerated an oral dose of 7.0 mg of zinc (as $ZnCl_2$) without showing any ill effects, but levels above this were fatal to some chicks. Normal chicks, however, could tolerate only 1.4 mg of zinc when injected intraperitoneally as an isotonic solution of $ZnCl_2$. Results of the zinc therapy are shown in table 7. The chicks receiving the oral zinc treatment died at about the same time as chicks receiving no treatment, and the percentage of survivors was about the same; the number of chicks receiving this treatment

TABLE 6

Tolerance of normal chicks to zinc chloride[1]

Intraperitoneal injection		Oral administration	
Zn	Survivors at 72 hours	Zn	Survivors at 72 hours
mg		*mg*	
14	0/5	14	2/6
7	6/16	7	5/5
5.6	6/16	1.4	5/5
4.2	0/5	0.7	5/5
2.8	1/10	0.14	5/5
1.4	14/15	0.07	5/5
1.3	4/5		
1.1	5/5		
0.7	5/5		
0.28	5/5		

[1] Treatment administered once approximately one day after hatching.

TABLE 7

Effects of zinc chloride therapy on survival of zinc-deficient chicks

Treatment	Survivors	Average survival of dead chicks
		hours
Injection, mg Zn		
1.4 (1×)	0/3	46
0.7 (1×)	1/4	72
0.7 (5×)	14/18	129
Oral, mg Zn		
2.8 (3×)	2/8	78
None	7/24	86

was small. These results suggest that zinc is rather poorly absorbed from the digestive tract of young chicks even if they are seriously deficient in this element. Injection of the maximal tolerable dose of zinc for normal chicks was fatal to the zinc-deficient chicks in a short time. Injection of 0.7 mg of zinc apparently was not effective as a single treatment; if this dose was given in 5 successive daily treatments, a much higher percentage of the chicks survived. The average survival time for those that died during the treatment period was increased. Chicks that survived both the deficiency and treatment periods, began to eat and drink and the deficiency symptoms disappeared. These chicks grew and after 4 to 5 weeks weighed approximately the same as the chicks from hens fed zinc-supplemented or casein diets. The only symptom that remained in these treated chicks was the frizzled appearance of the feathers (fig. 2), which was apparent until the feathers either broke off or were molted and replaced. The feathers appearing after the early set was molted were essentially normal. None of these chicks were maintained beyond 10 weeks of age; therefore, no data were available on their subsequent behavior.

Blood was drawn from some freshly hatched chicks of all groups and the blood hemoglobin concentration determined (table 5). The chicks from hens fed the low-zinc, soy protein diets were showing definite symptoms of zinc deficiency. With the significant ($P < 0.05$) exception of blood hemoglobin differences in chicks from hens fed the two calcium levels, maternal diet had no effect on blood hemoglobin concentration. This appears to contrast with the results of Norris et al. ('58) and O'Dell et al. ('58) which showed hemoconcentration in growing chicks that had developed zinc deficiency symptoms subsequent to normal hatching from hens fed a complete breeding hen diet. Perhaps effects of food and water intake on hemoconcentration that occurred in such chicks was not to be reflected in the present work.

It can be inferred from the first experiment that the hen's requirement expressed as parts per million of the total diet for zinc in an isolated soybean protein diet is less than the chick's requirement. In this study 18 ppm of zinc were adequate for normal embryonic development. O'Dell et al. ('58), Norris and Zeigler ('58), Roberson and Schaible ('58) and others have reported a zinc requirement of 30 to 35 ppm of zinc for chicks fed isolated soybean protein diets.

Deficiency symptoms observed in the newly hatched chicks from zinc-deficient hens were very simliar to those described by O'Dell et al. ('58) and Norris and Zeigler ('58) for older chicks made zinc-deficient after hatching. The only difference was the lack of hemoconcentration in the newly hatched chicks.

This experiment did not demonstrate an effect on zinc requirement due to the source of dietary protein. As discussed previously, however, an adequate comparison between casein and soy protein could not be made because of difference in the dietary zinc content. Excess dietary calcium aggravated the zinc deficiency, which is in agreement with some other studies of calcium and zinc interactions in the nutrition of swine and poultry reviewed by Forbes ('60).

Experiment 2. Egg production differences were not as great as in the first experiment (fig. 3), but again showed a decreased egg production when low-zinc soy protein diets were fed. As contrasted with the previous year, increased calcium level appeared to increase egg production irrespective of zinc level in the diet. Again, no zinc deficiency symptoms were apparent in the hens fed low-zinc diets for 6 months.

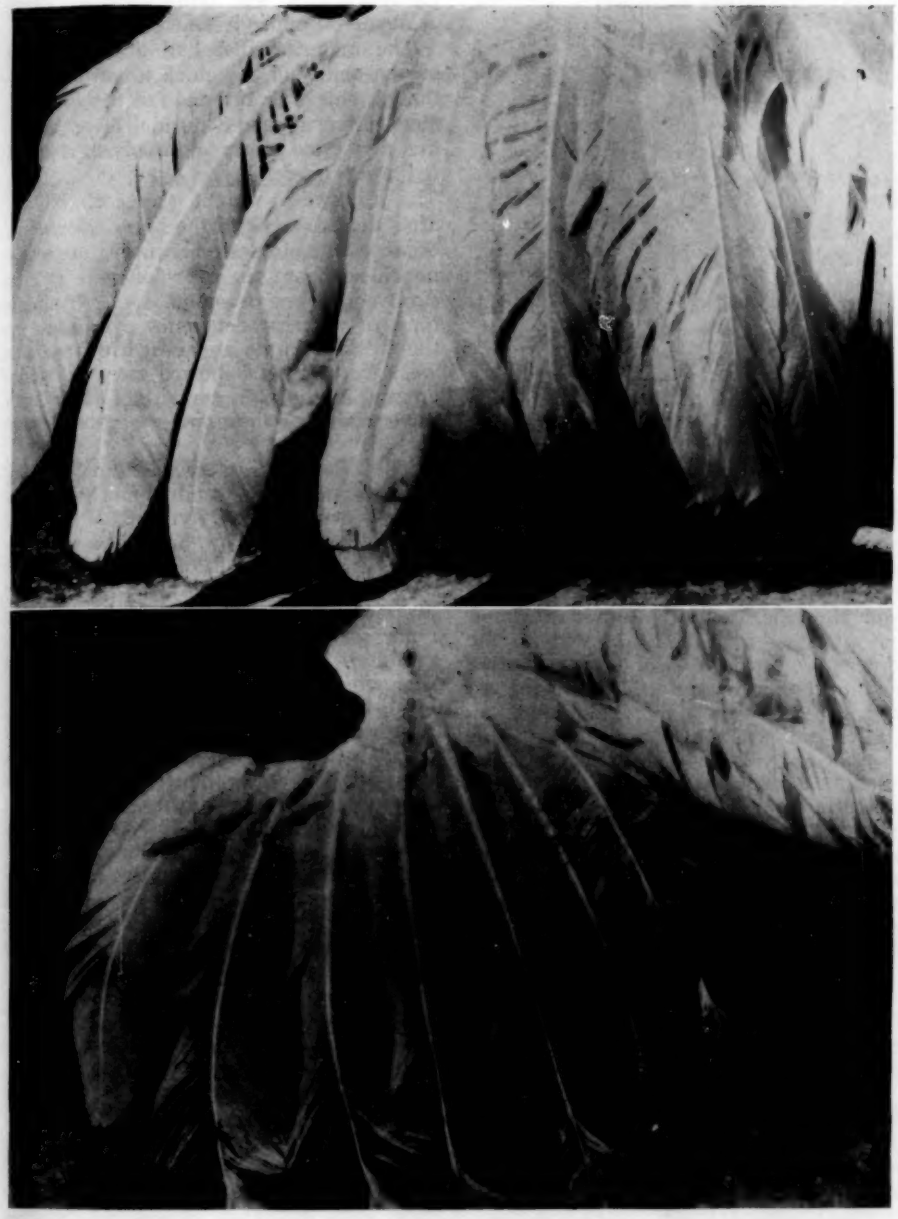

Fig. 2 Experiment 1. Effect of zinc in maternal diet on resultant feather growth of the chick. Feathers of representative 6-week-old chicks from hens fed the zinc-supplemented (top) and the zinc-deficient diets (lower). Chicks were fed identical diets after hatching.

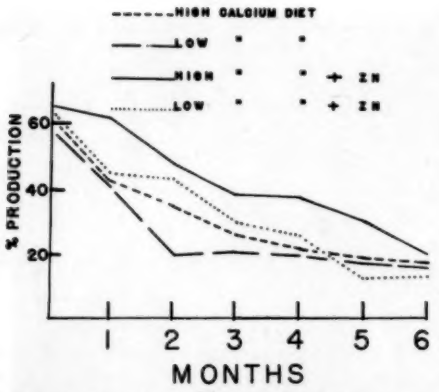

Fig. 3 Experiment 2. Effect of zinc on egg production from two dietary calcium levels. Percentage of production is plotted against time interval of the experiment.

Zinc content of yolks decreased from above 23 ppm of zinc early in the experiment to below 18 ppm of zinc (fresh-weight basis) in all of the eggs assayed from hens fed zinc-deficient diets. Hens producing the greatest number of abnormal embryos produced yolks with a zinc content as low as 5 ppm. This reduction in zinc content of the yolk is in agreement with a recent report by Blamberg et al. ('60). Tabulation of the results of zinc assay of egg yolk showed a wide variation among individual hens in the rate of zinc depletion from the yolk. Also the amount of zinc in eggs laid after hens had been fed the low-zinc diet for 6 months, varied widely between hens. The level of calcium did not appear to affect the concentration of zinc in the yolks. The concentration of zinc in yolks from hens receiving supplemental zinc was remarkably constant at about 30 ppm (fresh-weight basis).

The maternal diet had a great effect on the ability of the chick to survive. Although chicks from hens fed the low-zinc diets, often appeared normal after hatching, they grew poorly and many died when fed a commercial-type chick diet (table 8). First-week weight gains of chicks, after the 6th week of the hen experiment, became meaningless because practically no chicks from the zinc-deficient groups survived to one week of age. Although most of the zinc deficiency symptoms in the chicks became apparent after the 6th week, certain hens showed more rapid decreases in the zinc content of their eggs and produced abnormal embryos before the 6th week.

The effect of dietary calcium level on production of abnormal embryos is presented in figure 4. Only 13 days were required to produce the first abnormal embryo with the high-calcium diet, whereas 36 days were required with the normal level of calcium. Twenty-nine abnormal embryos (16.5% of all fertile eggs) were produced by hens fed the high-calcium diet, whereas only 14 such embryos (8.4% of all fertile eggs) were produced by hens fed the low-calcium diet. Ninety per cent of all hens (9 of 10 hens) receiving the high-calcium diet produced one or more abnormal embryos during the 6-month period compared with 50% of the hens receiving the low-calcium diet (5 out of 10). No embryo abnormalities were noted when identical diets were supplemented with an additional 55 ppm of zinc. These results again lead to the conclusion that a

TABLE 8

First week's growth of chicks as hatching season progressed (exp. 2)

Hen diet varied as follows		Growth the first week[1]		
Calcium	Zinc	2nd week hatch	4th week hatch	6th week hatch
%	*ppm*	*gm*	*gm*	*gm*
4.00	10	19 (20)[2]	12 (12)	3 (2)
2.25	10	16 (16)	16 (15)	13 (2)
4.00	65	16 (16)	16 (13)	16 (14)
2.25	65	17 (19)	16 (11)	20 (9)

[1] Eggs were set to hatch weekly.
[2] Numbers in brackets show number of chicks included in each average weight.

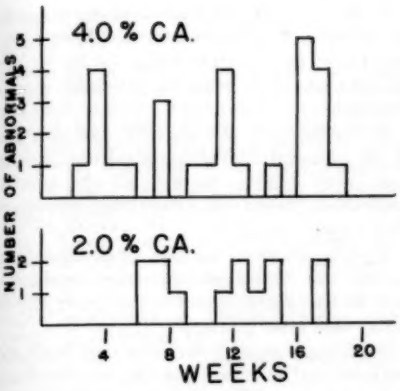

Fig. 4 Experiment 2. Effect of calcium in maternal hen diet on production of abnormal chick embryos. The number of abnormal embryos hatched each week is plotted against time interval of the experiment. (A total of 14 abnormal embryos from 168 fertile eggs (8%) and 29 abnormal embryos from 176 fertile eggs (16%) was observed from hens receiving the 2.25% and 4.0% calcium diets, respectively.)

high-calcium level in a maternal diet of the kind used intensified zinc deficiency symptoms in chick embryos.

Examples of abnormal embryos were photographed; two of these are included here (fig. 5). As was typical, both embryos lived until the latter stages of incubation. Since visceral tissues were normal in all abnormal embryos observed, the viscera were removed so that skeletal abnormalities might be viewed more clearly. The embryo with limbs exhibits micromelia, dorsal curvature of the spine, and abnormally shortened and fused thoracic and lumbar vertebrae. This embryo had two toes on each lower limb, whereas normal chicks have 4 toes. An edematous swelling of the neck muscles was also commonly observed. The other photograph shows an embryo with no lower skeleton or limbs; again the viscera were normal. Many kinds of structural abnormalities were observed involving head, limbs and vertebrae. Many of the first abnormal embryos were "rumpless" (absence of caudal vertebra and associated structures). In addition to skeletal disorders, eyes were sometimes absent or underdeveloped.

The abnormal embryos observed appeared to be similar in many respects to

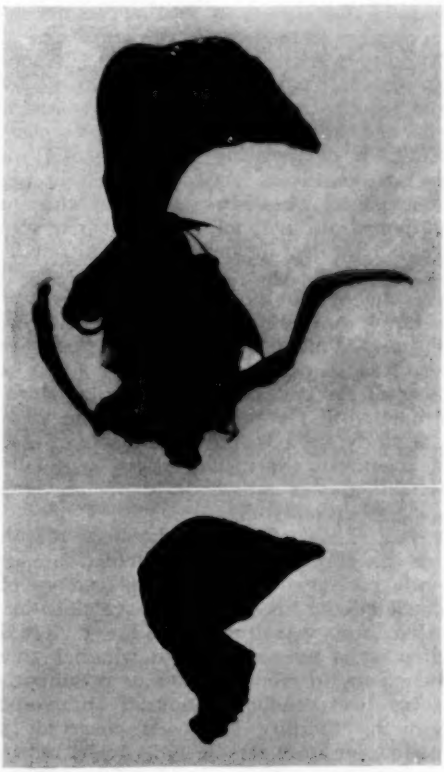

Fig. 5 Experiment 2. Typical abnormal embryos from hens fed a zinc-deficient diet. Both embryos were alive at normal hatching time (21 days' incubation). Skin is removed to better expose the total skeleton of each individual. Upper embryo exhibits phocomelia, micromelia, lordosis, shortened abnormal thorax and pelvic skeleton, and edematous pipping muscle. The lower embryo shows extreme ectrodactyly, and absence of any skeleton posterior from the cervical vertebra. Eye was also very underdeveloped.

those caused by insulin injection into the egg during incubation or a lack of those vitamins and minerals (Landauer, '48) needed for carbohydrate metabolism.

Landauer ('48) found that eggs injected with insulin before the third day of incubation developed into normal chicks except that the tail structures were missing (rumplessness). When eggs were injected after the third day, insulin caused abnormal smallness of limbs (micromelia). Insulin-induced rumpless embryos developed

exactly the same way as the genetic abnormality, termed recessive rumplessness. Another worker, Zwilling ('48), demonstrated that hypoglycemia was caused by the insulin injection into the egg, which resulted in micromelia or rumplessness. Addition of pyruvate to the insulin injection greatly reduced the resultant abnormality. This was evidence that a carbohydrate metabolism disturbance was caused by insulin and resulted in abnormality of the skeleton. It has been well established that zinc is an integral part of a number of enzymes using DPN as a coenzyme (Vallee, '59); certain of these are involved in carbohydrate metabolism. In view of the widespread occurrence of micromelia and rumplessness in the present study, the possible relationship of zinc deficiency to the insulin effect, especially as it affects carbohydrate metabolism, merits further study.

Regardless of the biochemical reason for the abnormality, some defects apparently prevented the normal placement of cartilagenous tissue prior to calcification when zinc was limited in the maternal diet. It is not now known whether zinc deficiency in other animals or in humans could also produce abnormal embryonic growth. Sterility has been observed in male laboratory rats severely restricted in dietary zinc (Millar et al., '58). Other aspects of reproduction, however, especially with diets not so severely limited in zinc, have not been investigated. The role of zinc requires further study in this respect.

SUMMARY

Two experiments, conducted with White Leghorn hens housed to exclude zinc from the environment and fed semipurified diets, led to the following conclusions:

1. Young hens receiving isolated soy protein-type diets (10 ppm of zinc) failed to gain normally unless the diet contained the higher levels of calcium (4.0%) and zinc (65 ppm) simultaneously. Unless supplemented with zinc, neither normal-calcium (2.25%) nor high-calcium (4.0%) isolated soy protein-type diets permitted normal egg production or hatchability. The chicks and developing embryos showed marked effects of zinc deficiency.

2. Increasing the zinc content of an isolated soybean protein-sucrose diet containing 4.0% of calcium from 10 to 18 ppm of zinc enabled hens to produce normal chicks.

3. Increasing the dietary calcium level of an isolated soybean protein-sucrose ration from 2.25% to 4% of the diet for hens potentiated the zinc deficiency in the developing chick.

4. The excess calcium appeared to decrease the blood plasma zinc concentration in the hens, but did not alter the red blood cell zinc content.

5. Blood hemoglobin levels of hens were not affected, but hemoglobin levels of zinc-deficient day-old chicks hatched from eggs laid by these hens were significantly ($P < 0.05$) lowered by increasing calcium in maternal diet.

6. Zinc-deficient chicks sometimes hatched, but were weak and would not stand, eat or drink. These chicks had an accelerated respiratory rate and showed labored breathing. These symptoms were aggravated by disturbance of the chicks and usually were fatal. Retarded feathering and frizzled feathers were observed. Repeated injections of zinc chloride greatly benefited such chicks, but chicks showed limited tolerance to such injections.

7. Embryonic development of chicken embryos was altered by withholding zinc from the maternal diet. The major defect was grossly impaired skeletal development.

8. Hens receiving small quantities of zinc in the diet deposited less zinc in the yolk than similar birds receiving supplemental zinc in otherwise identical diets.

9. Supplementation of maternal diet with 55 ppm of zinc as zinc carbonate prevented all abnormal embryonic development.

ACKNOWLEDGMENT

We are indebted to Dr. Thomas B. Roos, Department of Zoology, University of Wisconsin, for the suggestion that insulin injection produces abnormalities similar to those seen here.

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Effect of Vitamin D on the Utilization of Zinc, Cadmium and Mercury in the Chick[1]

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It has been recognized for many years that vitamin D plays an important role in the absorption of calcium (Migicovsky and Nielson, '51; Keane et al., '56). In a recent communication (Worker and Migicovsky, '61), we presented evidence indicating that in the chick, vitamin D may also play a part in the absorption of other elements of the Group IIA classification, namely, beryllium, magnesium, strontium and barium. Subsequently, we have extended these investigations and have examined the possible effects of vitamin D on the utilization of the Group IIB elements, zinc, cadmium and mercury. The results of these studies are reported in this communication.

MATERIALS AND METHODS

Preparation and dosing of chicks. The chicks used in the present experiments were New Hampshire/Rhode Island Red cross. They were obtained at one day of age, put into experimental batteries indoors, and fed a low-calcium, vitamin D-deficient ration previously described (Worker and Migicovsky, '61) until rachitic symptoms appeared, usually at three to 5 weeks of age. The birds were divided at random into two groups, and one group was dosed orally with 2000 IU of vitamin D_3 per bird in a colloidal solution. The dosed birds also received vitamin D_3 in the diet at the rate of 1000 IU per 100 gm of feed. Approximately 36 hours later the supplemented and non-supplemented groups were further subdivided into smaller treatment groups containing 15 to 20 birds. The birds were then dosed orally or subcutaneously with Zn^{65}, Cd^{115} or Hg^{203} in the chloride form. The radioactive dose in each case was only a fraction of a microcurie. Since the specific activities of the preparations used were high (50 to 100 mc per gm of element), the total mineral dose administered in each case was extremely small, namely, 10 μg per bird.

Measurement of radioactivity. The chicks were killed by chloroform anesthesia 24 hours after dosing with isotope. The left tibia was removed, cleaned and dried at 80° overnight. The bones were dissolved individually in 6 ml of concentrated HNO_3 and the solutions made up to a final volume of 10 ml. The gamma activity of these solutions was measured in a scintillation well counter as described previously (Worker and Migicovsky, '61).

RESULTS

In table 1 is summarized the effect of a vitamin D supplement on the levels of radioactivity appearing in bone following administration of the isotopes orally and subcutaneously. The data indicate that in the first experiment of the series (exp. 136), in which the isotopes were given orally, dosing with vitamin D caused a highly significant increase in the levels of zinc and cadmium in bone but had no effect on the level of mercury.

In the next experiment (exp. 137), in which the effect of vitamin D on the utilization of zinc given both orally and subcutaneously was investigated, significantly higher levels of radioactivity in the bones

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TABLE 1

Effect of vitamin D_3 on levels of radioactivity in chick tibia following administration of isotopes orally and subcutaneously

Exp. no.	Isotope	Dose	Route of administration	Vitamin D_3 treatment		Significance of difference P
				Non-supplemented	Supplemented	
		cpm × 10^3		cpm/tibia	cpm/tibia[1]	
136	Zn^{65}	390	oral	4560 ± 310	5700 ± 250	< 0.01
	Cd^{115}	475	oral	420 ± 42	620 ± 36	<< 0.01
	Hg^{203}	310	oral	380 ± 78	365 ± 55	N.S.
137	Zn^{65}	222	oral	930 ± 160	2420 ± 250	< 0.01
	Zn^{65}	111	subcutaneous	2200 ± 190	2060 ± 300	N.S.
139	Zn^{65}	108	oral	660 ± 88	1080 ± 42	< 0.01
	Zn^{65}	54	subcutaneous	920 ± 96	900 ± 108	N.S.
	Cd^{115}	400	oral	470 ± 68	830 ± 54	< 0.01
	Cd^{115}	100	subcutaneous	310 ± 55	285 ± 84	N.S.

[1] Mean ± standard error of mean.

of supplemented birds were observed when the isotope was dosed orally. When the isotopes were administered by the subcutaneous route, however, no significant differences in bone counts were observed between supplemented and nonsupplemented birds.

In the final experiment (exp. 139), the effects of vitamin D on zinc, dosed orally and subcutaneously, were again confirmed. In agreement with earlier findings, vitamin D supplementation also increased cadmium levels in bone when the isotope of this element was administered by the oral route. Vitamin D was observed to have no effect, however, when the isotope was given by the subcutaneous route. The pattern of behavior observed for cadmium appeared to parallel closely that noted for zinc.

DISCUSSION

The present experiments were undertaken to investigate the possible effects of vitamin D on the utilization of the Group IIB elements, zinc, cadmium and mercury. In an earlier communication from this laboratory (Worker and Migicovsky, '61), we reported evidence in the chick that vitamin D, besides its well known effect on calcium metabolism, markedly influenced the uptake of other elements of the Group IIA series: beryllium, magnesium, strontium and barium. These studies were therefore planned with a view to extending our earlier investigations to include the remainder of the Group II elements.

The results from the present studies indicate that vitamin D supplementation significantly increased bone counts of both zinc and cadmium after oral administration of isotopes of these elements to rachitic chicks. These observations provide good grounds for believing that, along with its generally recognized role in calcium metabolism and its more recently observed effect on other Group IIA elements, vitamin D also plays a part in the utilization of both zinc and cadmium. That vitamin D was effective in increasing bone levels of these elements when they were administered orally but not when dosed subcutaneously would further suggest that, as has been shown previously in the case of calcium (Migicovsky and

Jamieson, '55) and other elements of the Group IIA series (Worker and Migicovsky, '61), the influence of vitamin D was at the site of absorption. Further work, however, is clearly required to confirm this observation beyond doubt.

The results obtained with mercury are too limited to draw any firm conclusions. At the time these experiments were undertaken, our supplies of chicks were very limited, a situation which precluded further studies with this isotope. On the basis of the present results, however, the possibility is suggested that, in contrast with its effect on the uptake of zinc and cadmium, vitamin D plays little or no part in the movement of mercury.

Further studies on the interrelationships of vitamin D and mineral uptake are continuing. The foregoing results have shown that vitamin D may, under certain conditions, increase the uptake of both zinc and cadmium in the chick. To our knowledge this is the first suggestion in the literature that vitamin D may be involved in the metabolism of either of these elements. The practical significance of these findings still remains to be elucidated. It remains to be seen also whether members of other periodic groupings are similarly affected. The current findings, along with those of earlier studies on the Group IIA elements (Worker and Migicovsky, '61), provide an indication, at least, that this effect may be a more general one than has previously been believed.

SUMMARY

Studies were reported on the effect of vitamin D on the metabolism of zinc, cadmium and mercury after administration of isotopes of these elements orally and subcutaneously to rachitic chicks.

The movement of zinc and cadmium from an oral dose into bone was significantly increased by vitamin D, (no such increase was found for mercury).

When zinc and cadmium were dosed subcutaneously, no difference in concentration of isotopes in bone between supplemented and nonsupplemented birds was observed.

These findings suggest that vitamin D may exert an effect on the metabolism of both zinc and cadmium and that the site of this effect is in the absorption mechanism.

Attention is drawn to the similarity of the results obtained with zinc and cadmium in these experiments and those obtained previously with calcium and other elements of the Group IIA series.

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Problems in the Prediction of Protein Values of Diets: Caloric Restriction

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Protein malnutrition in man is frequently associated with low caloric intakes of foods which may have an adequate protein value if consumed in unrestricted amounts—because energy requirements are satisfied preferentially to protein requirements. This factor must be taken into account in the evaluation of dietary survey data and in the prescription of modifications to poor regimens.

The protein value of diets fed ad libitum may be calculated from a knowledge of the amino acid composition and the concentration of the protein they contain (Miller and Payne, '61a, b). The total energy of food eaten daily, however, is also important, for if this is reduced below a certain level an increasing amount of protein is burned for energy purposes, and, consequently, the efficiency of utilization of protein decreases (Platt et al., '61). Thus Forbes and Yohe ('55) reported no change in the biological value of a diet when the food intake of rats was reduced from 8 to 6 gm per day, but a decrease from 99 to 69 when the food intake was further reduced to 4 gm per day.

Under conditions of caloric restriction, the protein value of a diet will depend upon the energy available for protein anabolism rather than on the concentration and the nature of the protein it contains. Thus, one might expect for any given diet a range of food intakes over which NDpCals % (net dietary protein calories %, Platt and Miller, '59) is constant and below which it falls with the caloric intake.

Let C = caloric intake; C_B = calories required for basal metabolism; then $C - C_B$ = calories available for other purposes. Let E = calories required for the synthesis of 1 Cal. of protein.[1]

Where protein synthesis is limited by caloric intake the protein calories retained by an animal are given by

$$\frac{C - C_B}{E} \qquad (1)$$

and if the percentage of protein calories in the diet is P, the intake of protein calories is given by

$$\frac{P \times C}{100} \qquad (2)$$

Miller and Payne ('61b) define net protein utilization (NPU) as:

$$\text{NPU} = \frac{\text{Retained N}}{\text{Intake N}} = \frac{\text{Protein calories retained}}{\text{Intake protein cals.}}$$

From 1 and 2:

$$\text{NPU} = \frac{100\ (C - C_B)}{\text{PCE}}$$

and

$$\text{NDpCals \%} = \text{NPU} \times P = \frac{100}{E}\left(1 - \frac{C_B}{C}\right) \qquad (3)$$

This equation applies only where the synthesis of protein is limited by the energy available, as above this level NDpCals % depends upon protein concentration and "score" (Miller and Payne, '61a, b). This is illustrated in figure 1.

For practical purposes it remains to obtain some estimate of the constants in equation 3. Brody ('45) stated that the requirement for basal metabolism (C_B) is 70 Cal. per day per kg of body weight to the power of 0.73, for all species from mice to elephants: this gives a value for C_B.

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[1] E includes not only the stored energy of the protein but also the work aspect to growth "analagous to that expended for rearranging the chairs in a room, in which case the potential energy of the chairs or room is not increased." (Brody, '45).

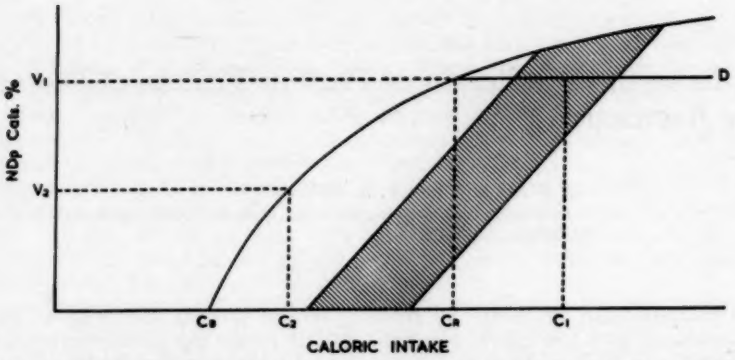

Fig. 1 The diagram illustrates the effect of caloric intake on NDpCals %. When fed ad libitum the caloric intake of animals fed diet D will generally fall within the shaded area (e.g., C_1) and NDpCals % $= V_1$. If the intake is reduced to C_R the NDpCals will still be V_1, but if it is further reduced to C_2 the value will have fallen V_2. The curve is given by $\text{NDpCals} = \frac{100}{E}(1 - \frac{C_B}{C})$ where C = caloric intake, C_B = calories required for basal metabolism, and E = calories required for the synthesis of one Cal. of protein.

No direct evaluation of E is to be found in the literature, but the results presented in this paper suggest a value of about 6 Cal. per protein calorie.[2] A value of this magnitude is consistent with the work of Rubner ('08).

Inserting these values in equation 3:

$$\text{NDpCals \%} = 17\ (1 - \frac{70}{C}) \qquad (4)$$

where C is the caloric intake measured as calories per day per kilogram of body weight to the power of 0.73.

Six diets were fed over a wide range of caloric intakes and the measured protein values plotted together with the curves predicted from equation 4 (fig. 2).

METHODS

The methods used in these investigations were the same as reported previously (Miller and Payne, '61a). Food intake was controlled by feeding once daily, limited amounts of food which were expressed as calories per kilogram of body weight to the power of 0.73. NPU was determined by the method of Miller and Bender ('55) as modified by Miller and Payne ('61a).

RESULTS

Analytical data for the 6 diets used are given in table 1. Three of the diets (E. and W. Pakistan, and Persian) are derived from food survey data. One contained 10% of casein (10C), one was a stock diet (Am) and the 6th was a mixture containing 20% of the stock diet diluted with the non protein diet (20 Am). The semi-synthetic diets were made according to Miller and Bender ('55).

In figure 2 are presented the observed values for NDpCals of these diets fed over a wide range of caloric intakes. Each of the curves represents predicted values for a diet, the curved portion being derived from equation 4 and the linear portion representing the mean NDpCals % over the ad libitum range of caloric intake.

The curved portion could be derived statistically by using those results which fall below the mean NDpCals % over the ad libitum range of caloric intakes, and plotting them against the reciprocal of the caloric intake. The method gives a value for E = 6.2 and $C_B = 66$.

DISCUSSION

Protein malnutrition in man is frequently associated with inadequate caloric intakes. The assessment of the protein value of diets under these conditions is

[2] A value of 6.2 is obtained from a statistical examination of the data.

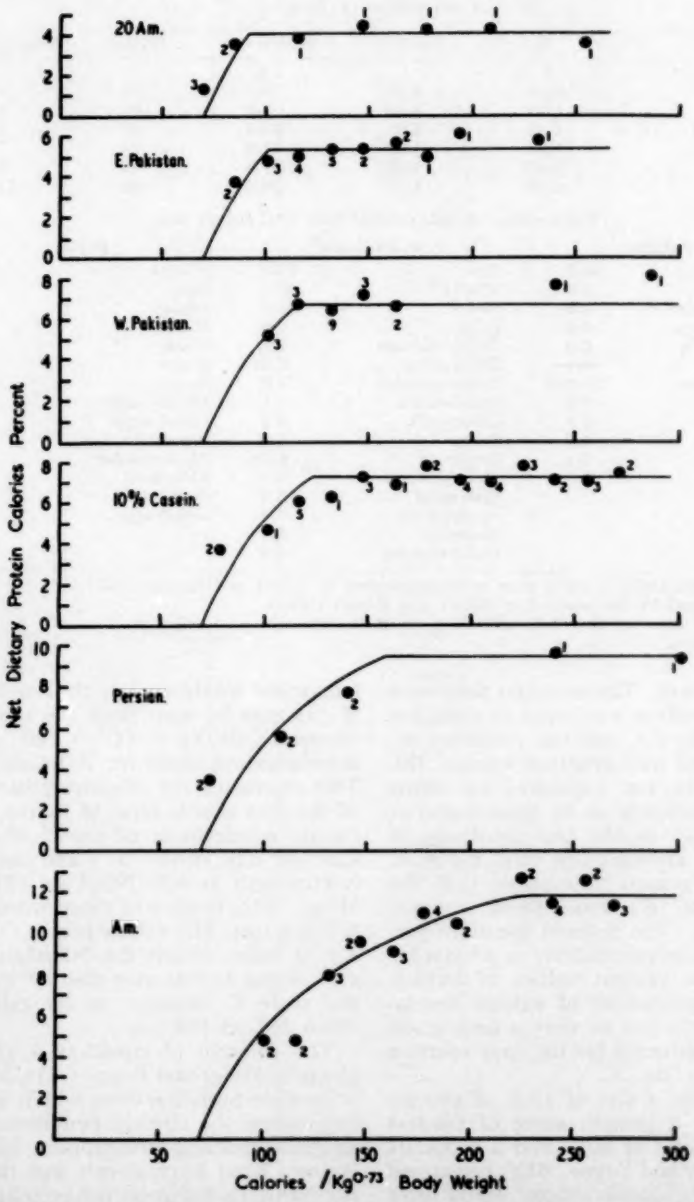

Fig. 2 The net dietary-protein value of 6 diets fed at various levels of caloric intake. The numerals show the number of independent trials made at each level of intake; each replicate consisted of 4 rats treated as a single group.

TABLE 1

Composition of diets[1]

Diet	N	Cal./gm	Protein Cal.	NPU_{st}[2]	NDpCals %[3]
	%		%		
Am, 20%[4]	0.83	4.10	5.1	78	4.0
E. Pakistan	1.49	4.16	9.0	62	5.3
W. Pakistan	2.30	4.32	13.3	57	6.8
Casein, 10%	1.80	4.00	11.3	73	7.3
Persian	2.76	4.00	17.3	72	9.5
Am[4]	4.13	4.10	25.2	80	11.9

Composition of diets derived from food survey data

E. Pakistan		W. Pakistan		Persia	
Parboiled rice	90.6	Rice	17.2	Wheat	59.2
Pulse	3.9	Wheat	52.9	Rice	6.3
Potato powder	0.8	Maize	3.3	Barley	9.7
Dried cabbage	0.4	Pulse	4.6	Maize	1.0
Fish meal	0.9	Dried cabbage	2.2	Pulse	4.8
Dried egg	0.1	Dried apple	0.04	Sugar	4.9
Meat powder	0.003	Potato powder	4.0	Butter	1.0
Dried milk	0.5	Dried swede	0.1	Dried swede	2.4
Mustard oil	1.8	Dried milk	3.1	Dried apple	2.4
Sugar	1.0	Butter	4.4	Almonds	0.5
Dried apple	0.1	Dried egg	0.05	Meat powder	3.9
		Meat powder	1.4	Fish meal	0.5
		Fish meal	0.4	Dried milk	2.9
		Mustard oil	0.4	Dried egg	0.5
		Sugar	5.1		
		Curry powder	0.9		

[1] The semisynthetic diets were made according to Miller and Bender ('55).
[2] Calculated by the method of Miller and Payne ('61a).
[3] Mean values for the diets when fed ad libitum.
[4] Stock diet.

clearly important. The equation presented in this paper offers a solution to a similar problem in the rat, and the predicted results agree well with observed values. The caloric intakes are expressed in terms which are applicable to all species and at least one (C_s) of the two constants in equation 4 is known to be valid for man. There is no reason to suppose that the other constant (E) would show any species variation. The authors therefore present this equation tentatively as a basis for predicting the protein values of human diets under conditions of caloric restriction. With this end in view a nomogram has been constructed for the easy solution of equation 4 (fig. 3).

For example, a diet of 18% of protein calories, and a protein score of 66 has NPU (operative) of 50% and a NDpCals of 9% (Miller and Payne, '61b) consumed at an adequate caloric intake, would meet the protein requirements of young children. If fed to a child of 10 kg at the rate of only 650 Cal. per day, however, the protein value would be less than 9 NDpCals %, as may be seen from the nomogram, whence $Cals/Kg^{0.75}$ (C) = 120 and the corresponding value for NDpCals % = 7. This represents the effective protein value of the diet at this level of intake. As the caloric requirement of the child is 1000 Cal. per day (FAO, '57) and the protein requirement is 8% NDpCals (Platt and Miller, '59), neither of these requirements is being met. The caloric intake (150 $Cal./Kg^{0.75}$) below which the NDpCals of this diet begins to fall may also be read from the scale C adjacent to its value of 9 when fed ad libitum.

The solution of equation 4 and those given by Miller and Payne ('61a, b) makes it possible to define diets which are likely to produce the clinical conditions known as marasmus and kwashiorkor. Miller and Payne ('61a) have shown that diets having NDpCals 4% meet the protein requirements for maintenance; and Brody ('45) states that the caloric requirement for maintenance is twice that for basal me-

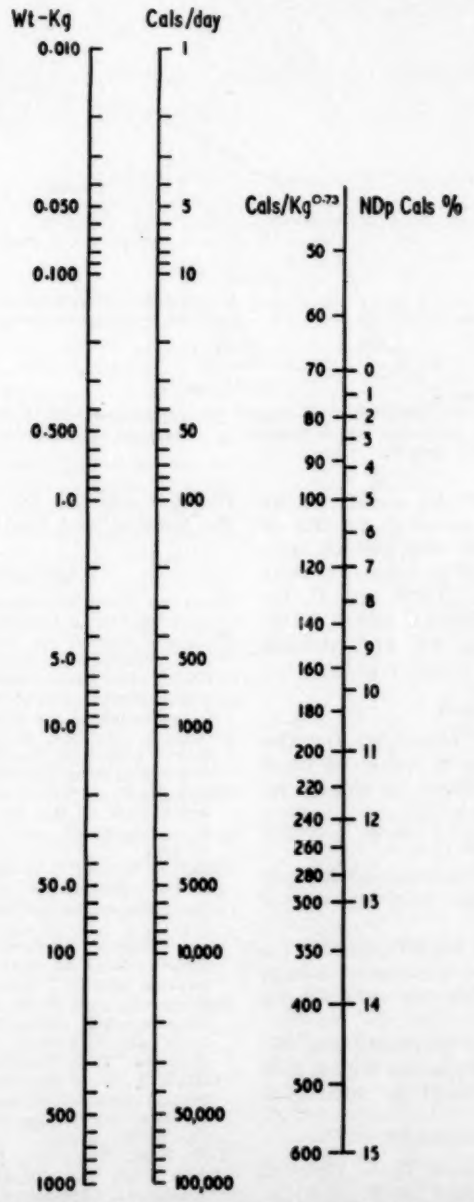

Fig. 3 If a ruler is laid between the points corresponding to the caloric intake per day and body weight in kilograms, values for calories per day per $Kg^{0.73}$ (C) and NDpCals % may be read off. The value for NDpCals % obtained in this way may be compared with the value of the diet when fed ad libitum (or calculated by the method of Miller and Payne, '61b). The lesser of the two values is the effective protein value of the diet.

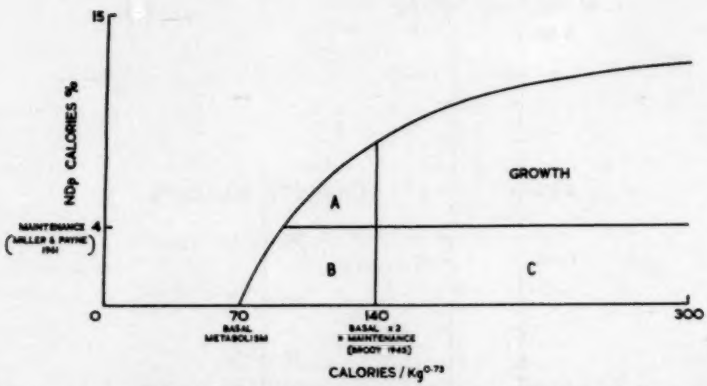

Fig. 4 Four areas of conditions with respect to caloric and protein supplies are shown. Growth is possible in one area, but is limited by caloric intake in areas A and B and by NDpCals % in areas B and C.

tabolism. These two limits are shown in figure 4 and define 4 areas of conditions with respect to calorie and protein supplies, three of which fail to support growth for different reasons: (1) A and B, insufficient calories; and (2) C and B, NDpCals % too low. The 4th area defines conditions which will support growth.

SUMMARY

1. An equation is presented for the prediction of the protein value of diets when fed under conditions of caloric restriction:

$$\text{NDpCals \%} = 17\ (1 - \frac{70}{C})$$

where C is the caloric intake per day per kilogram of body weight to the power of 0.73.

2. Observed values for NDpCals % for 6 diets fed to rats over a range of caloric intakes agree well with the values from the equation.

3. A nomogram is presented for the easy solution of the equation which it is proposed tentatively could be applied to man.

ACKNOWLEDGMENT

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Two Alfalfa Factors with Cellulolytic Activity for Rumen Microorganisms

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The presence of unidentified factors in alfalfa that increase feed utilization and stimulate cellulolytic activity of rumen microorganisms has been postulated by several investigators (Burroughs et al., '50a, '50b, '50c; Ruf et al., '53; Bentley et al., '54). These factors have been difficult to concentrate in sufficient quantities for determining ruminant requirements. Dehority et al. ('57), using the artificial rumen technique, reported the isolation and identification of leucine, isoleucine, valine, and proline in alfalfa extracts as agents that increased cellulose digestion.

The presence of a factor in alfalfa leaf meal required for maximal growth of the mold, *Neurospora sitophila*, was reported by Novak et al. ('53). This substance was recently concentrated one-thousand-fold, by Novak et al. ('58), using this mold as a test organism for evaluating and comparing the degree of activity in the various fractions.

The purpose of this study was to isolate cellulolytic factors which might be present in the concentrates prepared by Novak et al. ('58) and to show that they are distinct from those isolated by Dehority et al. ('57).

EXPERIMENTAL

Concentration procedure. The method used for concentrating the cellulolytic factors from dehydrated alfalfa leaf meal is shown in figure 1. The detailed technique from the initial HCl extraction to the charcoal adsorption and elution is discussed by Novak et al. ('58). The fuller's earth eluate (pH adjusted to 7.0) was passed through an activated charcoal (Darco G-60) chromatographic column, and the active factors were eluted with 0.1 N HCl (125 ml of acid per 25 gm of alfalfa equivalents). The Darco eluate was evaporated to dryness on a steam bath, and the residue adjusted to volume with distilled water (1 ml equivalent to 1.6 gm of alfalfa equivalents).

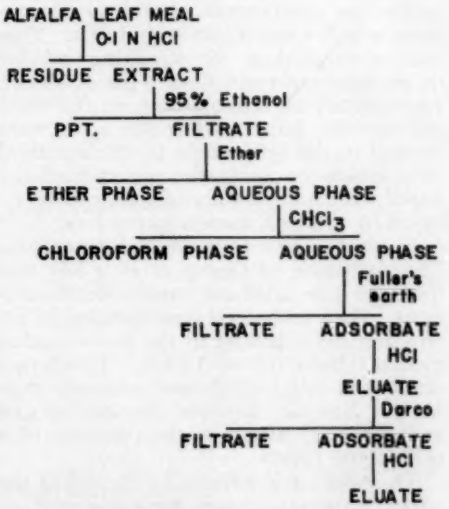

Fig. 1 Fractionation scheme of cellulolytic factors from alfalfa meal.

Chromatographic method. The Darco eluate was streaked on sheets of Whatman no. 1 chromatographic paper (13.5 cm, width). The chromatograms were resolved with butanol, acetic acid, and water (4:1:1) for 18 to 20 hours at room temperature. They were air-dried and the zones detected by the standard strip technique using a 0.2% acetone solution of ninhydrin. This was performed to locate the zones above and below proline, since Dehority et al. ('57) have accounted for the activity from the zone above proline which contained the amino acids proline, valine, leucine, and isoleucine. The paper

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was cut into the upper and lower zones; the upper zone consisted of compounds which migrated from proline to the solvent front; the lower zone, those which migrated from the base line to proline. The compounds of both zones were eluted with warm 0.1 N HCl, and aliquots of each zone were rechromatographed to confirm that a true separation had been attained.

After observing a significant increase in cellulose digestion resulting from the eluates of the lower zone, it was necessary to locate the compounds on the chromatogram which caused this stimulation. This was accomplished by streaking minute quantities (not more than 1 gm of alfalfa equivalents) of Darco eluate on the chromatographic paper. After the spots were located in the lower zone by the standard strip technique, each was eluted from the paper with 0.1 N HCl and the activity assayed by artificial rumen techniques.

In vitro rumen fermentation technique. The procedure of Cheng et al. ('55) was used for the artificial rumen determinations. Their technique was modified by increasing the cellulose in the fermentation medium from 0.5 to 1.25%. The larger amount of cellulose allowed a greater margin for digestion between the control and supplemented media in the presence of a cellulolytic factor.

The upper and lower zone eluates of the paper chromatograms were assayed at levels ranging from 0.4 to 3.2 gm of alfalfa equivalents. In order to narrow the range of maximum stimulation, the lower zone was also assayed at 6 levels ranging from 0.2 to 1.6 gm of alfalfa equivalents. The eluates of individual spots were then supplemented to the medium at a rate of 1 gm of alfalfa equivalents. All levels were determined in triplicates, using a fresh supply of alfalfa extract for each experiment.

RESULTS AND DISCUSSION

A summary of the results of preliminary experiments conducted to determine whether cellulolytic activity was evident from compounds present in the Darco eluate and lower zone of the paper chromatogram is shown in table 1. These results show that activity was obtained from all three supplements. The stimulation resulting from the addition of eluates from the lower zone of the chromatogram supports the assumption that the fractionation technique employed concentrated cellulolytic factors which were not present in the alfalfa fractions discussed by Dehority et al. ('57). They reported essentially no activity from the lower zone of their paper chromatogram. Six ninhydrin-positive spots were observed when the eluate of the lower zone was rechromatographed. The same amino acids (leucine, isoleucine, valine, and proline) isolated by Dehority et al. ('57) also were identified in the upper zone. The increases produced by the supplements were statistically significant at the 1% level (Duncan, '55).

TABLE 1

Cellulolytic activity of chromatographic fractions from extracts of alfalfa meal

Supplements to basal medium[1]	Cellulose digested[2]
	%
None	30.2
Darco eluate	52.8[3]
Lower zone	43.3[3]
Upper zone	40.5[3]

[1] Added at a level of 0.8 gm of alfalfa equivalents.
[2] The average of triplicates.
[3] Statistically significant ($P < 0.01$).

Since this investigation was conducted with factors obtained directly from a natural source, it was assumed that the concentration of these substances would vary among samples of alfalfa. Consequently, it was necessary to determine the point of maximum stimulation obtained by the addition of eluates from the lower zone of the chromatograms. A knowledge of the quantity of eluate needed to cause this degree of stimulation would then be assurance of an adequate range to compensate for the varying factor concentrations among test samples of alfalfa. The results of this study are reported in table 2. Values in experiment 1 indicated that the range of maximum stimulation existed between levels of 0.4 and 1.6 gm of alfalfa equivalents. In experiment 2, these supplementation levels were narrowed, and the range was evident between 0.8 and 1.2 gm. Maximum digestion (53.1%) occurred with 1-gm equivalents. The increases in cellulose digestion at the maximum point of stimulation were statistically significant (1% level) in both experiments.

TABLE 2

Cellulolytic activity of upper and lower zones from paper chromatograms

Supplements to basal medium[1]	Cellulose digested[2]			
	Lower zone		Upper zone	
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
	%	%	%	%
None	15.0	22.1	34.8	25.6
0.2	—	30.7	—	—
0.4	25.2	34.7	35.0	29.9
0.8	30.2[3]	36.4	36.7	25.7
1.0	—	53.1[3]	—	—
1.2	—	33.2	—	—
1.6	22.9	34.7	37.8	31.9
2.0	12.1	—	37.0	32.5
2.4	5.5	—	36.8	30.1
3.2	1.4	—	38.7	29.5

[1] Expressed as grams of alfalfa equivalents per tube.
[2] Average of triplicate samples.
[3] Statistically significant ($P < 0.01$).

Response in cellulose digestion obtained from assay tubes supplemented with eluates from the upper zone were not as pronounced as those from the lower zone. Values reported in table 2 indicate that the stimulation curves were irregular and inconsistent. Analysis of the data shows that these increases were not statistically significant.

These data indicate that the fractionation technique used to concentrate the cellulolytic stimulants, appearing in the lower part of the chromatogram, resulted in the removal of the amino acids leucine, isoleucine, valine, and proline to such an extent that at their reduced concentration they could not cause the high rate of stimulation evidenced in the scheme proposed by Dehority et al. ('57). This decrease in concentration of these amino acids from the upper zone was confirmed by a study of the intensity of the ninhydrin spots. These spots decreased in intensity from the initial acid extraction to the column separations.

Cellulolytic activity of the spots isolated from the lower zone of the paper chromatogram is shown in table 3. These results indicate that stimulation is due to compounds 5 and 6. These increases in cellulose digestion were statistically significant at the 5% level. The R_f values for these substances show that they are slow-moving on the chromatogram, at least slower than the common amino acids. It was also observed that these two spots required several hours for development of the ninhydrin color, whereas the common amino acids developed fairly rapidly. Eluates of the lower zone were also assayed with the test mold, *N. sitophila*, and it was found that compounds 5 and 6 (table 3) were the only ones which showed increased growth above the control. This suggests that both factors, which cause a stimulation of growth for the test organism, are the same factors responsible for the increase in cellulose digestion, *in vitro*.

TABLE 3

Cellulolytic activity of chromatographic spots in the lower zone

Spots	R_f value[1]	Cellulose digested[2]
		%
Control	—	24.2
1	0.48	22.4
2	0.40	11.9
3	0.25	14.5
4	0.19	26.8
5	0.12	33.6[3]
6	0.06	32.9[3]

[1] For butanol, acetic acid, and water descending chromatogram (4:1:1).
[2] Average of triplicate samples.
[3] Statistically significant ($P < 0.05$).

Lee et al. ('61) recently isolated two unidentified compounds from alfalfa meal which behave similarly, both chromatographically and as stimulants for the test mold, as the cellulolytic factors isolated in this investigation. Using a Dowex 50 (H^+) chromatographic column for the isolation, they observed that on paper chro-

matograms, these stimulants moved between known samples of L-lysine and L-cystine and did not behave chromatographically as did any of the common amino acids. They further observed that the two factors were evident as ninhydrin-positive spots only after hydrolysis of a water extract of alfalfa meal, suggesting their presence in bound form.

SUMMARY

Two cellulolytic factors from alfalfa meal have been isolated. The technique involved acid-extraction of alfalfa leaf meal, precipitation of the extract with ethanol, extraction of the ethanol filtrate with ether and chloroform, adsorption and elution of the solvent insoluble fraction on fuller's earth and Darco. Paper chromatography was used to separate ninhydrin-positive compounds present in the Darco eluate. These compounds were eluted from the chromatograms and tested for cellulolytic activity by *in vitro* rumen techniques.

Significant increases in cellulose digestion were attributed to the Darco eluate and to two ninhydrin-positive substances isolated from this eluate. On a paper chromatogram the active compounds moved more slowly than most of the common amino acids, migrating between known samples of L-lysine and L-cystine. They exist in bound form in alfalfa meal but can be released by acid hydrolysis.

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Metabolic Interrelationships Between Vitamin B_{12} and Pantothenic Acid in the Rat[1]

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Interrelationships between pantothenic acid and vitamin B_{12} in the nutrition of different species of animals have been reported (Evans et al., '51; Yacowitz et al., '51; Welch and Couch, '54; Balhoun and Phillips, '57). Boxer et al. ('53) observed a fivefold increase in the coenzyme A concentration of liver in vitamin B_{12}-deficient chicks. Further studies with rats, although confirming the earlier observations, revealed that increases were also observable in the kidney, although not in the brain (Boxer et al., '55). It was also evident that the increase was due neither to a decreased destruction of coenzyme A in the deficient tissues, nor to a shift in the ratio of the oxidized to the active reduced form of coenzyme A (Boxer et al., '55). Similar observations have since been reported by others (Sanguinetti et al., '56; Wong and Schweigert, '56) and it has been suggested that since the vitamin B_{12}-deficient animal cannot utilize carbohydrate efficiently (Ling and Chow, '54), the increase in liver coenzyme A may be a physiological adaptive mechanism that increases energy production by providing more two carbon fragments from fatty acid oxidation. A similar deranged carbohydrate metabolism exists in diabetic animals and the impaired energy production appears to be offset by an elevation of liver coenzyme A stores.[2] Cold stress observed to produce a vitamin B_{12} deficiency (Ershoff, '53) also causes an increase in coenzyme A levels (Campbell et al., '60).

An increase in liver concentration of vitamin B_{12} in pantothenic acid deficiency, first reported by Radhakrishnamurty and Sarma ('57), and confirmed by several others (Okuda, '57; Moruzzi et al., '58; Aiyar et al., '59) is also attended by increases in serum vitamin B_{12} and in urinary excretion of vitamin B_{12}.[3]

The reported increase in betaine-homocysteine transmethylase activity in pantothenic acid deficiency (Ericson and Harper, '55) and a decrease of the same in vitamin B_{12} deficiency (Oginsky, '50; Williams et al., '53; Mistry et al., '55; Ericson et al., '56) lends further support to the reciprocal nature of the relationship existing between the two vitamins.

In view of the reported metabolic relationships between pantothenic acid and methionine (Ludovici et al., '51; Dinning et al., '54, '55), vitamin B_{12} and methionine (Stekol and Weiss, '50; Bennett, '50; Fox et al., '59; Moruzzi et al., '60) and between the two vitamins themselves, it was thought worthwhile to study the metabolism of coenzyme A and certain related sulfhydryl compounds, in an attempt to elucidate the mechanism of the increased hepatic coenzyme A concentration in vitamin B_{12} deficiency.

Observations on the changes in tissue levels of coenzyme A, glutathione and total soluble sulfhydryl in simple deficiencies of vitamin B_{12} produced by feeding either a high vegetable protein diet or a purified casein ration devoid of the vitamin, and on the kinetics of *in vivo* biosynthesis of coenzyme A in rats with a single deficiency of vitamin B_{12} and with double deficiencies of vitamin B_{12} and pantothenic acid, from intraperitoneally administered precursors are presented and discussed.

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[2] Tompkins, G. Quoted as personal communication, in Novelli, G. D. 1957 Ann. Rev. Biochem., *26*: 249.

[3] Aiyar, A. S., and A. Sreenivasan, unpublished observation.

EXPERIMENTAL

Induction of deficiencies. (a) A simple vitamin B_{12} deficiency was produced by feeding weanling male rats (Wistar strain), weighing 45 to 50 gm, either a maize-groundnut meal diet (Fatterpaker et al., '59), or a purified 10% casein ration (Fatterpaker et al., '60) devoid of vitamin B_{12} for 8 weeks.

Control groups were also maintained with a supplement of vitamin B_{12} (200 µg/kg) to the respective basal diets.

(b) A double deficiency of pantothenic acid and vitamin B_{12} was produced in one group of rats by feeding the purified casein ration devoid of both calcium pantothenate and vitamin B_{12} for 8 weeks, by which time, the animals exhibited severe symptoms of deficiences of both vitamins.

Four animals each from the groups fed the vitamin B_{12}-deficient and vitamin B_{12}-replete casein rations, were given a supplement of L-cysteine hydrochloride (0.1 gm per kg of diet), throughout the experimental period of 8 weeks.

Administration of vitamin B_{12} to deficient rats. To 8 rats made deficient in vitamin B_{12} by maintenance with the basal purified casein ration, vitamin B_{12} (10 µg) was administered intraperitoneally and the animals were sacrificed at intervals of zero, 4, 8, 16 and 48 hours from the time of administration.

In vivo biosynthesis of coenzyme A in vitamin B_{12}-deficient rats. Vitamin B_{12}-deficient and vitamin B_{12}-supplemented rats were injected intraperitoneally with 10 mg each of calcium pantothenate and cysteine hydrochloride and were sacrificed at intervals of zero and 8 hours. Intraperitoneal injection of vitamin B_{12} (10 µg) was given to one group of deficient rats three hours prior to administration of the precursors.

In vivo biosynthesis of coenzyme A in rats deficient in pantothenic acid and vitamin B_{12}. To rats deficient in both the vitamins, L-cysteine hydrochloride (10 mg) was injected with or without prior administration (three hours prior) of either calcium pantothenate (10 mg) or vitamin B_{12} (10 µg) and the animals were sacrificed 8 hours later.

Determinations. The animals were sacrificed at the end of the experimental periods by decapitation, and the livers, after perfusion with isotonic saline, were excised and chilled in cracked ice. The livers were accurately weighed and made into 10% homogenates in isotonic sucrose (0.25 M) using a Potter-Elvehjem type glass homogenizer fitted with a Teflon pestle.

Total soluble sulfhydryl (Grunert and Phillips, '51) and methionine (Horn et al., '46) were determined colorimetrically essentially as described by the authors and glutathione was determined by the procedure outlined by Kasbekar and Sreenivasan ('59).

TABLE 1

Changes in coenzyme A and related sulfhydryl compounds in rat liver in vitamin B_{12} deficiency[1]

Group	Vitamin B_{12}	Total soluble sulfhydryl	Glutathione	Total methionine	Pantothenic acid	Coenzyme A
	mµg	mg	mg	mg	µg	units
Maize-groundnut meal diet	47±7[2]	1.02±0.11	0.90±0.08	5.07±0.13	131±16	165±21
Maize-groundnut meal diet+ vitamin B_{12}	92±19	1.28±0.03	1.15±0.14	5.21±0.01	81±13	94±14
10% Casein diet	49±4	1.14±0.07	0.88±0.03	4.98±0.09	120±12	151±14
10% Casein diet+ vitamin B_{12}	87±9	1.26±0.14	0.99±0.07	5.26±0.11	66±19	87±9

[1] Weanling male rats (45 to 50 gm) were reared with either a maize-groundnut meal diet or a purified casein ration deficient in vitamin B_{12}. Where indicated, vitamin B_{12} was supplemented at 200 µg/kg. Determinations were as detailed in text.

[2] Results are averages of 4 independent determinations ± standard error of the mean and are expressed per gram of fresh liver.

Vitamin B_{12} and pantothenic acid were assayed microbiologically using *Euglena gracilis* (Hoff-Jorgensen, '54) and *Lactobacillus arabinosus* (Skeggs and Wright, '44), respectively, as the test organism.

Coenzyme A was assayed using the acetylating enzyme from pigeon liver by the method of Kaplan and Lipman ('48).

RESULTS AND DISCUSSION

Observations on changes in the hepatic stores of coenzyme A and related sulfhydryl compounds in vitamin B_{12} deficiency are presented in table 1. Vitamin B_{12} deficiency produced by feeding either the high vegetable protein diet or the purified casein ration resulted in a similar increase in coenzyme A concentration of liver, attended by a decrease in total soluble sulfhydryl, glutathione and methionine.

Cysteine supplementation of the 10% casein diet, which is low in methionine, resulted in further increases in the coenzyme A levels with insignificant changes in total soluble sulfhydryl and glutathione in the vitamin B_{12}-deficient group. In the vitamin B_{12}-supplemented group, however, the increases in total soluble sulfhydryl and glutathione were more than the increase shown in coenzyme A (table 2).

In table 3 are presented data on the effects of administration of a single dose of vitamin B_{12} intraperitoneally to deficient animals. Significant increases in total soluble sulfhydryl, glutathione and methionine were observed by the end of 8 hours after administration of the vitamin with practically no change in the coenzyme A level. By the end of 16 hours coenzyme A level showed a decline and was consider-

TABLE 2

Changes in liver stores of coenzyme A due to L-*cysteine supplementation of the diet*[1]

Group	Total soluble sulfhydryl	Glutathione	Total methionine	Total pantothenic acid	Coenzyme A
	mg	*mg*	*mg*	*μg*	*units*
10% Casein diet	1.14 ± 0.07[2]	0.88 ± 0.06	4.98 ± 0.07	120 ± 12	151 ± 16
10% Casein diet + L-cysteine	1.21 ± 0.03	1.07 ± 0.07	5.23 ± 0.09	143 ± 7	179 ± 7
10% Casein diet + vitamin B_{12}	1.26 ± 0.02	0.99 ± 0.07	5.26 ± 0.02	66 ± 11	87 ± 13
10% Casein diet + vitamin B_{12} + L-cysteine	1.30 ± 0.04	1.19 ± 0.11	5.29 ± 0.10	87 ± 13	99 ± 19

[1] L-Cysteine (100 mg/kg diet) was supplemented to both the vitamin B_{12}-deficient and vitamin B_{12}-replete casein diets throughout the experimental period of 8 weeks.

[2] Results are averages of 4 independent determinations ± standard error of the mean and are expressed per gram of fresh-weight liver.

TABLE 3

Effect of administration of vitamin B_{12} *to deficient rats on liver levels of coenzyme A*[1]

Hours after administration	Total soluble sulfhydryl	Glutathione	Total methionine	Coenzyme A
	mg	*mg*	*mg*	*units*
0	1.14 ± 0.07[2]	0.88 ± 0.10	4.98 ± 0.07	151 ± 16
4	1.21 ± 0.01	0.97 ± 0.02	5.11 ± 0.11	148 ± 11
8	1.24 ± 0.03	1.03 ± 0.05	5.14 ± 0.09	147 ± 12
16	1.29 ± 0.08	1.14 ± 0.09	5.10 ± 0.02	139 ± 19
48	1.29 ± 0.03	1.14 ± 0.02	5.17 ± 0.09	113 ± 15

[1] Vitamin B_{12} (10 μg/rat) was administered intraperitoneally to the deficient animals and sacrificed at intervals of zero, 4, 8, 16 and 48 hours.

[2] Results are averages for duplicate samples of liver for each of the groups of 8 rats ± standard error of the mean, and are expressed as per gram of fresh-weight liver.

TABLE 4

Biosynthesis of coenzyme A in vivo in vitamin B_{12}-deficient rats[1]

Hours after administration	Total soluble sulfhydryl	Glutathione	Total methionine	Coenzyme A
	mg	*mg*	*mg*	*units*
		10% Casein diet		
0	1.14 ± 0.03[2]	0.88 ± 0.07	4.98 ± 0.09	151 ± 9
8	1.19 ± 0.06	0.97 ± 0.03	4.96 ± 0.03	191 ± 17
8[3]	1.27 ± 0.02	1.08 ± 0.10	5.13 ± 0.02	164 ± 13
		10% Casein diet + vitamin B_{12}		
0	1.26 ± 0.10	0.99 ± 0.02	5.26 ± 0.06	87 ± 10
8	1.29 ± 0.09	1.17 ± 0.07	5.29 ± 0.04	101 ± 12

[1] Calcium pantothenate (10 mg) and cysteine hydrochloride (10 mg) were administered intraperitoneally and the animals sacrificed at intervals of zero and 8 hours.

[2] Results are averages of 4 independent determinations ± standard error of the mean and are expressed per gram of fresh weight liver.

[3] Vitamin B_{12} (10 μg) injected intraperitoneally three hours prior to administration of the precursors.

ably reduced by 48 hours, whereas glutathione and methionine show a gradual rise.

Data on the *in vivo* biosynthesis of coenzyme A from intraperitoneally administered precursors in vitamin B_{12}-deficient and vitamin B_{12}-supplemented rats are presented in table 4. The biosynthesis of the coenzyme occurred more in the deficient group than in the supplemented group in which the synthesis of glutathione and total soluble sulfhydryl appeared greatly enhanced. Administration of vitamin B_{12} three hours prior to administration of L-cysteine hydrochloride and calcium D-pantothenate to the vitamin B_{12}-deficient animal resulted in reduction in the biosynthesis of coenzyme A with attendant increases in the synthesis of glutathione, total sulfhydryl and methionine.

A combined deficiency of vitamin B_{12} and pantothenic acid resulted in low hepatic coenzyme A levels and slightly decreased total soluble sulfhydryl and glutathione levels (table 5). Administration of L-cysteine hydrochloride led to a slight increase in the levels of total soluble sulfhydryl. Prior (three hours) administration of calcium pantothenate effected increased synthesis of coenzyme A, and of vitamin B_{12} favored increased synthesis of total soluble sulfhydryl and glutathione.

The results point to an increased channeling of cysteine into coenzyme A rather

TABLE 5

Biosynthesis of coenzyme A in vivo in rats deficient in pantothenic acid and vitamin B_{12}[1]

Compounds administered			Total soluble sulfhydryl	Glutathione	Total methionine	Coenzyme A
Vitamin B_{12}[2]	Calcium pantothenate[2]	L-Cysteine[3]				
			mg	*mg*	*mg*	*units*
—	—	—	0.93 ± 0.03[4]	0.81 ± 0.09	4.77 ± 0.09	94 ± 11
—	—	+	1.21 ± 0.04	0.84 ± 0.03	4.73 ± 0.01	91 ± 13
—	+	+	1.07 ± 0.08	0.86 ± 0.06	4.73 ± 0.07	137 ± 18
+	—	+	1.31 ± 0.06	1.11 ± 0.03	5.01 ± 0.10	99 ± 7

[1] Weanling rats were maintained with a purified 10% casein ration devoid of both vitamin B_{12} and pantothenic acid for 8 weeks.

[2] Where indicated vitamin B_{12} (10 μg) and calcium pantothenate (10 mg) were administered parenterally three hours prior to L-cysteine hydrochloride.

[3] L-Cysteine hydrochloride (10 mg/rat) was administered intraperitoneally and the animals sacrificed 8 hours later.

[4] Results are averages of 4 independent determinations ± standard error of the mean and are expressed per gram of fresh weight liver.

than into glutathione or methionine in the vitamin B_{12}-deficient rat, possibly due to the reported participation of vitamin B_{12} in the biosynthesis of glutathione (Kasbekar et al., '59) and in the formation of methionine (Oginsky, '50) from cysteine through homocysteine. The increase in coenzyme A is possibly a metabolic adaptation necessitated by the impaired carbohydrate metabolism, for more effective concentration of the coenzyme to participate in fatty acid oxidation, in the vitamin B_{12}-deficient animal (Wong and Schweigert '56).

SUMMARY

1. The elevation in hepatic coenzyme A in the vitamin B_{12}-deficient rat was attended by decreases in total soluble sulfhydryl, glutathione and total methionine. The changes were reversed and the levels returned to almost normal values within 48 hours after administration of a single dose of vitamin B_{12}.

2. Supplementation of a low-methionine diet with L-cysteine hydrochloride resulted in increases in liver stores of coenzyme A, total soluble sulfhydryl, glutathione and methionine, the rise in coenzyme A level being more in the vitamin B_{12}-deficient rat than in the supplemented one.

3. The vitamin B_{12}-deficient animal showed greater *in vivo* synthesis of coenzyme A from intraperitoneally administered precursors, than the vitamin-supplemented animal. Prior administration of vitamin B_{12} to the deficient animal decreased the coenzyme A synthesis.

4. Administration of L-cysteine hydrochloride to rats deficient in both pantothenic acid and vitamin B_{12} was without appreciable effect on the liver levels of coenzyme A, total soluble sulfhydryl, glutathione and methionine. Prior administration of pantothenic acid or of vitamin B_{12} favored increased synthesis of coenzyme A or of total soluble sulfhydryl, glutathione and methionine, respectively.

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Effects of Varied Levels and a Single Daily Supplement of Lysine on the Nutritional Improvement of Wheat Flour Proteins[1]

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Since the studies of Osborne and Mendel ('14), numerous investigations have shown that the nutritional value of various incomplete proteins may be improved by the addition of the limiting amino acid. Consequently, considerable interest has been aroused in the fortification of protein-containing foods with amino acids.

It has been observed that the level and the method of amino acid supplementation exert a profound effect on body function. The studies of Harper et al. ('55a) demonstrated that an excess of dietary leucine increased the isoleucine requirement of the young rats fed a low-protein diet. The growth depression, which occurred in the young rats receiving a rice diet supplemented with excessive amounts of lysine and threonine, was prevented by the addition of certain other amino acids (Harper et al., '55b). Elvehjem and Harper ('55) concluded that the supplementation of a diet with a single amino acid may seriously affect the requirement for other amino acids.

The studies of Henry and Kon ('46), Schaeffer and Geiger ('47) and Geiger ('47, '48) indicated that the tissue anabolism in the animal body occurred most efficiently when all of the essential amino acids were supplied simultaneously. Henderson and Harris[2] observed that a lysine supplement, given to rats fed a lysine-deficient diet, was not efficiently utilized if given more than three hours later than the rest of the diet. Geiger ('50) concluded that the effective supplementation of the missing amino acid is possible only if the time interval is less than 4 or 5 hours, when there are still sufficient quantities of the deficient protein in the intestine of the animal. Relatively little is known, however, of the utilization of a single daily supplement of the amino acid administered apart from the diet.

Therefore, experiments were conducted to study the effect of graded levels of lysine supplement on the nutritive value of the wheat flour proteins for the young rats, and to study further whether the lysine supplement administered apart from the diet was as well utilized by the young rats as the same amount of supplement incorporated in the diet.

EXPERIMENTAL

Male weanling rats of the Sprague-Dawley strain, about 21 days old and weighing from 50 to 60 gm, were used. The animals were individually housed in suspended wide-mesh wire cages in an air-conditioned room maintained at 24°C and 40% of relative humidity. During a 4-week experimental period, deionized water was given ad libitum to all the animals, while the food allowances among groups were varied.

Three experiments were carried out. Experiment 1 was conducted to study the effect of the graded levels of lysine supplement on the nutritional value of the wheat flour proteins. The rats were fed ad libi-

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[2] Henderson, R., and R. S. Harris 1949 Concurrent feeding of amino acids. Federation Proc., *8*: 382 (abstract).

tum a flour diet or the same diet supplemented with various levels of lysine by the addition of appropriate quantities of L-lysine hydrochloride.[3] The percentage composition of the flour diet was as follows: enriched white bread flour,[4] 82; corn oil, 8; salt (Jones and Foster, '42), 4; cornstarch, 3; nonnutritive bulk,[5] 2, and vitamin-cornstarch (Yang et al., '59), 1. The lysine supplement was added to the diets at the expense of an equal amount of cornstarch. The flour diet contained approximately 10% of protein (N × 6.25) and 1.19% of L-lysine.[6] The lysine content was determined by a microbiological assay using *Leuconostoc mesenteroides* P-60 and the medium developed by Steele et al. ('49).

Experiments 2 and 3 were conducted to study whether the lysine supplement administered by stomach tube in a single daily dose was as well utilized as the same amount of supplement included in the diet. The flour diet supplemented with 0.2% of lysine, which supported the optimal weight gain in experiment 1, was chosen for this study. The diet contained a total of 0.39% of L-lysine (0.19% from flour). To facilitate the administration of the lysine given apart from the diet, an aqueous lysine solution was prepared so that each milliliter contained 0.02 gm of lysine, which was the amount of the supplemental lysine in 10 gm of the flour diet supplemented with 0.2% of lysine. The amount of the lysine solution given to the rats was adjusted daily so that it was equivalent to the average amount of the supplemental lysine consumed by the animals receiving the lysine supplement incorporated in the diet. The animals receiving no lysine solution were given an equivalent amount of deionized water by stomach tube to equalize the effect of handling.

In experiment 2, the food allowances among groups were equalized. The amount of food given to the animals receiving the lysine supplement, either in the diet or by the stomach tube, was restricted to the average amount of the food consumed on the previous day by the animals receiving the unsupplemented flour diet. The lysine solution or the deionized water was given at 9 AM daily, when the daily quota of the food was offered.

Experiment 3 was conducted to determine the effect of the delayed lysine supplementation on the nutritive value of the flour proteins. Rats were trained during an 8-day pretest period to consume their daily diets within a 4-hour period. They were then divided according to body weight into comparable groups and given ad libitum the flour diet or the same diet supplemented with 0.2% of lysine for 4 hours daily, from either 9 AM to 1 PM (part A) or 5 PM to 9 PM (part B). At the end of the 4-hour feeding period, or 4, 8, 12, or 16 hours thereafter, the animals receiving the unsupplemented flour diet were given by stomach tube a quantity of lysine solution equivalent to the average amount of the supplemental lysine contained in the food eaten on the same day by the animals fed the supplemented flour diet. The animals receiving the lysine-supplemented flour diet were given an equivalent amount of deionized water by stomach tube immediately after the feeding period.

Food intakes and body weight were recorded for all rats. Measurements of the apparent digestible nitrogen and the biological value were carried out during the last 7 days on experiment by procedures previously described (Yang et al., '59). Livers and the carcasses of the animals in experiment 1 were also analyzed for the dry-matter, nitrogen and lipid content. Data of the experiments were subjected to the analysis of variance (Snedecor, '56) and Duncan's multiple range test (Duncan, '55).

RESULTS AND DISCUSSION

Supplementation of the flour diet with lysine, up to the level of 0.5%, significantly ($P < 0.01$) increased the food intake (table 1). The animals given the

[3] Nutritional Biochemicals Corporation, Cleveland.

[4] General Mills, Inc., Minneapolis. The flour was enriched with the following supplements in mg per 100 pounds of flour: thiamine mononitrate, 180; riboflavin, 90; niacin, 1200; and ferric phosphate 900.

[5] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[6] Determined by Mrs. Lois L. Reitz.

TABLE 1

Effects of feeding rats a wheat flour diet supplemented with various levels of lysine[1]

Lysine supplement	Food intake	Weight gain	Nitrogen efficiency[2]	Apparent digestible nitrogen[3]	Biological value[4]
%	gm	gm		%	%
0	198±19[5]	11± 3	3.6±0.7	88.6±0.7	34.4±4.9
0.06	245±23	30± 5	7.6±0.6	87.6±2.5	39.1±2.5
0.12	289±32	53± 7	11.3±0.5	88.1±1.1	48.1±3.4
0.20	294±19	59± 8	12.4±1.1	87.1±1.0	52.6±2.7
0.25	263±26	53± 8	12.2±1.0	86.4±0.6	55.0±2.4
0.50	235±24	46±11	11.6±1.3	87.4±1.9	54.5±1.6
1.00	215±15	41± 5	10.8±1.0	85.8±0.6	51.1±2.4

[1] Ten rats/group.

[2] Weight gain in grams/gram of nitrogen eaten.

[3] $\frac{\text{(Nitrogen in food} - \text{nitrogen in feces)} \times 100}{\text{Nitrogen in food}}$

[4] $\frac{\text{(Nitrogen in food} - \text{nitrogen in urine} - \text{nitrogen in feces)} \times 100}{\text{Nitrogen in food} - \text{nitrogen in feces}}$

[5] Mean ± standard deviation.

flour diet supplemented with 1.0% of lysine, however, did not consume significantly ($P > 0.05$) more food than those given the unsupplemented flour diet. All of the 6 tested levels of the lysine supplement significantly ($P < 0.01$) increased the weight gain. The maximal weight gain was noted in the group receiving the flour diet supplemented with 0.2% of lysine. Higher levels of the supplementation gave reduced weight gains, possibly due, in part, to the amino acid imbalance.

The mean nitrogen efficiency (grams gained per gram of nitrogen eaten) of the flour diet supplemented with 0.06% of lysine was significantly ($P < 0.01$) higher than that of the unsupplemented flour diet, but lower ($P < 0.01$) than the values of the flour diet with higher levels of lysine supplementation. The nitrogen efficiency values of the flour diet supplemented with 0.20 or 0.25% of lysine were not significantly different from the value of the flour diet with 0.5% of added lysine, but were significantly higher than that of the flour diet plus 1.0% of lysine.

The mean value for the apparent digestible nitrogen of the unsupplemented flour diet was not significantly different from the values of the flour diets supplemented with 0.06 to 0.5% of lysine, but greater ($P < 0.01$) than that of the flour diet supplemented with 1.0% of lysine.

The biological value of the flour proteins was increased, in a stepwise fashion, by the lysine supplementation up to the 0.25% level. Higher levels of the supplementation did not further increase the biological value, and in fact the diet supplemented with 1.0% of lysine had a lower biological value than those supplemented with 0.25 or 0.50% of lysine ($P < 0.05$).

Data on liver and carcass composition of the animals fed the various flour diets are summarized in table 2. The mean dry-matter content of the livers for the various groups ranged from 27.2 to 29.9%. The liver nitrogen levels tended to increase and the liver lipid levels tended to decrease as the level of the lysine supplementation increased. Harper ('56) has previously pointed out that the lysine deficiency causes fatty livers in the young rats. The present observations also confirm the findings of Harris and Burress ('59), who demonstrated that the lysine supplementation decreased the hepatic lipid level and increased the hepatic nitrogen level of the young rats fed a diet containing 8% of wheat proteins.

Dry-matter content of the carcasses of the various groups ranged from 29.7 to 33.2%. Data also indicated that the lysine supplementation of the flour diet increased the carcass nitrogen content. The highest carcass lipid level was noted in the group given the flour diet supplemented with 0.12% of lysine. Higher levels of supplementary lysine reduced the percentage of the carcass lipid content. Agreement was

TABLE 2

Dry matter, nitrogen and lipid[1] content of livers and carcasses of young rats fed a flour diet supplemented with various levels of lysine[2]

Lysine supplement	Livers			Carcasses		
	Dry matter	Nitrogen	Lipids[1]	Dry matter	Nitrogen	Lipids
%	%	%	%	%	%	%
0	28.3±2.2[3]	2.87±0.32	8.9±1.1	29.7±1.7	2.87±0.16	8.8±1.1
0.06	27.7±0.8	3.10±0.17	8.4±0.8	32.0±1.0	2.89±0.07	12.1±2.1
0.12	29.9±2.0	2.92±0.25	9.1±1.3	33.2±1.5	2.84±0.08	14.1±2.0
0.20	27.4±1.9	3.15±0.12	7.7±1.7	32.1±1.2	3.02±0.06	11.2±2.0
0.25	28.0±0.9	3.12±0.27	7.4±1.6	31.2±1.1	2.96±0.10	10.9±2.3
0.50	27.5±1.4	3.17±0.30	6.6±0.4	30.6±1.8	3.02±0.07	9.6±2.2
1.00	27.2±1.0	3.20±0.13	6.6±0.7	30.0±1.0	3.02±0.11	9.5±2.0

[1] Alcohol-ether-extract.
[2] Ten rats/group.
[3] Mean ± standard deviation.

TABLE 3

Effects of feeding rats a wheat flour diet with lysine supplement either mixed in the diet or administered apart from the diet[1]

Lysine supplement	Food intake	Weight gain	Nitrogen efficiency[2]	Apparent digestible nitrogen[3]	Biological value[4]
	gm	gm		%	%
None	226±23[5]	15±5	3.6±0.8	89.7±1.9	45.4±2.9
Mixed in diet	234±8	35±4	8.0±0.8	88.2±0.9	56.6±1.6
By stomach tube	234±5	33±3	7.6±0.9	88.9±1.4	57.9±1.9

[1] Ten rats/group.
[2] Weight gain in grams/gram of nitrogen eaten.
[3] See footnote 3, table 1.
[4] See footnote 4, table 1.
[5] Mean ± standard deviation.

noted between the carcass and the liver lipid levels, both of which were maximal in the animals given the diet containing 0.12% of the added lysine, and were reduced by higher levels of lysine supplementation.

Data on food intake, weight gain, nitrogen efficiency, apparent digestible nitrogen, and biological value obtained in experiment 2 and 3 are summarized in tables 3 and 4, respectively. When the food intakes were equalized at a level based on the average food consumption of rats fed the unsupplemented flour diet, the mean values of weight gain, nitrogen efficiency, and biological value obtained with the unsupplemented flour diet were significantly ($P < 0.01$) lower than those obtained with the diet supplemented with 0.2% of lysine either mixed in the diet or administered by stomach tube in a single daily dose (table 3). The mode of administration of lysine supplement had no significant effect on weight gain, nitrogen efficiency, digestible nitrogen, and biological value. These observations indicated that the lysine supplement given apart from the diet in a single daily dose was as well utilized by the young rats as the same amount of lysine supplment fed in the diet. It should be noted, however, that the food allowance for the animals receiving the lysine supplment by either method was adjusted to a suboptimal level and the lysine solution was administered at 9 AM, when the daily quota of the food was offered. Since the animals receiving a restricted food intake usually ate most of their food rapidly, it would appear reasonable to conclude that the animals received the lysine supplement by stomach tube and the flour proteins at approximately the same time. To determine the effect of the delayed lysine supplementation on the nutritive value of the flour proteins, further studies were conducted.

As shown in table 4, the values obtained for the food intake, weight gain, nitrogen

TABLE 4

Effects of feeding rats a wheat flour diet with delayed administration of lysine supplement[1]

Lysine supplement	Food intake	Weight gain	Nitrogen efficiency[2]	Apparent digestible nitrogen[3]	Biological value[4]
	gm	*gm*		%	%
		Part A			
Mixed in diet	200 ± 18[5]	38 ± 5	12.2 ± 0.9	82.7 ± 2.5	46.1 ± 5.2
By stomach tube					
Immediately after feeding	189 ± 13	34 ± 3	11.7 ± 0.6	84.7 ± 2.6	49.9 ± 5.6
4 Hours after feeding	198 ± 12	39 ± 1	12.4 ± 0.9	85.0 ± 2.0	53.0 ± 3.4
8 Hours after feeding	196 ± 21	41 ± 6	13.3 ± 0.9	84.0 ± 1.9	49.8 ± 3.2
		Part B			
Mixed in diet	217 ± 26	40 ± 6	12.3 ± 1.2	84.9 ± 3.1	47.6 ± 6.0
By stomach tube					
12 Hours after feeding	210 ± 18	37 ± 6	11.1 ± 1.1	83.0 ± 1.7	48.8 ± 4.5
16 Hours after feeding	208 ± 18	36 ± 4	10.9 ± 1.1	84.6 ± 2.9	47.0 ± 4.1

[1] Seven rats/group.
[2] Weight gain in grams/gram of nitrogen eaten.
[3] See footnote 3, table 1.
[4] See footnote 4, table 1.
[5] Mean ± standard deviation.

efficiency, apparent digestible nitrogen, and biological value when the lysine supplement was given apart from the diet immediately, 4, or 8 hours after the 4-hour feeding period, were not significantly ($P > 0.05$) different from those obtained when the lysine supplement was included in the diet (part A). In part B, when the lysine supplement was administered either 12 or 16 hours after the feeding, the values for the flour proteins were not statistically ($P > 0.05$) different from those observed when the lysine supplement was included in the diet (table 4).

Therefore, the lysine supplement administered apart from the diet once a day appears to be as well utilized as when incorporated in the diet. The studies of Geiger ('47), Schaeffer and Geiger ('47), and Henderson and Harris,[7] however, have shown that the amino acid supplements given apart from the diet several hours later were not utilized efficiently by the young rats; but the experimental procedures adopted by those investigators were different from those used in the present experiment. In their studies, the supplemental amino acid was incorporated into an otherwise adequate but protein-free diet, and was thus ingested simultaneously with the other components. In the present experiment, the supplemental amino acid was administered alone by stomach tube in a single daily dose. Further studies, therefore, are being conducted to compare the two methods of administering amino acid supplement to determine whether there is a significant difference in the results obtained.

SUMMARY

Experiments were conducted to study the effect of various levels of lysine supplementation on the nutritional value of the wheat flour proteins for the young rats, and to study further whether the lysine supplement administered apart from the diet once a day was as well utilized as the same amount of supplement incorporated in the diet.

Growth data and the biological value indicated that the nutritional value of the flour proteins was improved by the lysine supplementation up to a level of 0.20 or 0.25%, but that liver and carcass composition afforded little information of value at these levels. Certain adverse effects occurred when the flour diet was supplemented with 1.0% of lysine.

When food intakes were equalized at a suboptimal level, growth data and the biological value obtained with lysine supplementation by stomach tube were similar to those with the supplementation in the

[7] See footnote 2.

diet. Growth data and the biological value obtained with the lysine supplement administered apart from the diet, either immediately or 4, 8, 12 or 16 hours after the 4-hour feeding period, were not different from those observed with the lysine supplement incorporated in the diet.

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Effect of Hypocaloric Feeding on Gross Body Composition

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The general supposition that obesity is predisposing to a shorter life expectancy and leanness to a longer life expectancy is based mainly on actuarial data. These data strongly suggest that obesity shortens life span perhaps through its associated effects on the cardiovascular and renal system. Vital statistics are somewhat inadequate, however, as it is difficult to acquire basic information on genetic and environmental history of the individual. Therefore, to acquire baseline information on the various parameters which are associated with the "normal" aging process, it becomes necessary to seek this information within laboratory confines. McCay et al. ('35) in classical experiments showed that hypocalorically reared rats had a marked increase in life expectancy. More recently, Berg ('60) reported an increased life expectancy of rats that were calorically underfed but not as severely as those described by McCay ('35). The latter author described the rats as being very lean and healthy, although this description was subjective as no chemical composition studies were performed.

Yiengst et al. ('59) have shown that as animals grow older, the chemical composition of the body changes. Conrad and Miller ('56) have likewise reported changes in body composition with time in the rat. It seemed desirable, then, to establish whether the gross body composition of rats of different chronological ages would change if the rats were maintained on an undercaloric regimen which held their body weight at a constant level during the entire course of the testing period.

MATERIALS AND METHODS

Animals. Male rats of the Naval Radiological Defense Laboratory's (NRDL) Specific Pathogen-Free Sprague-Dawley strain were fed the diet described by McCay et al. ('35) at 21 days of age. The only modification of the diet was the addition of a vitamin B-complex supplement. When the rats started to receive the diet, they weighed 60 to 70 gm. The hypocalorically reared rats were allowed to grow at a rate such that they reached 125 gm at three months of age. It was found by experience that feeding 5 gm of the diet one day and 3 gm the next, the 125-gm weight could be attained during the three-month period. From then on, the 125-gm weight could be maintained as long as desired by feeding 5 gm per day. By contrast, rats fed this diet ad libitum attained an average weight of 450 gm at three months. The times selected for analysis of total body composition in these calorie retarded rats were 13 weeks (fed diet 10 weeks) and 23 weeks of age (fed diet 20 weeks). Analyses of total body composition was also carried out on a group of young weight controls that attained the 125-gm body weight after receiving the diet for about one week.

Sample preparation and analytical methods. Preparation of the carcass and the analytical methods used for the determination of ash, protein, water and fat were the same as those reported by Mickelsen and Anderson ('59).

RESULTS

Age, time fed the diet, and gross body composition of the rats in this experiment are presented in table 1. During the course of hypocaloric feeding such as that used here, total body fat decreased and total protein increased. The differences in the fat and protein shown in the results are highly significant at the 99% level. The mineral content increased during the

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TABLE 1

Relative body composition of hypocalorically reared rats at 13 and 23 weeks of age compared with young weight control rats

No. of rats	Age weeks	Weeks fed diet	Body weight	Body weight minus GI contents	Fat	Water	Protein	Ash
			gm	gm	%	%	%	%
5	5	1	124 ± 5.1[1]	110 ± 4.7	6.39 ± 0.40	73.0 ± 0.62	17.1 ± 0.27	2.8 ± 0.08
5	13	10	125 ± 0.0	125 ± 0.0	4.90 ± 0.94	72.2 ± 0.85	20.2 ± 0.27	3.1 ± 0.14
5	23	20	124 ± 0.58	124 ± 0.3	2.24 ± 0.06	72.2 ± 0.15	21.4 ± 0.13	4.1 ± 0.11

Body composition of hypocalorically reared rats at 13 and 23 weeks of age compared with young weight controls in absolute quantities

No. of rats	Age weeks	Weeks fed diet	Body weight	Body weight minus GI contents	Fat	Water	Protein	Ash
			gm	gm	gm	gm	gm	gm
5	5	1	124 ± 5.1[1]	110 ± 4.7	7.04 ± 0.58	80.2 ± 3.6	18.8 ± 0.9	3.1 ± 0.13
5	13	10	125 ± 0.0	125 ± 0.0	6.12 ± 1.2	90.3 ± 1.0	25.3 ± 0.35	3.82 ± 0.17
5	23	20	124 ± 0.58	124 ± 0.3	2.78 ± 0.07	89.7 ± 0.62	26.6 ± 0.12	5.12 ± 0.18

[1] The values in this table represent means ± S.E.

course of the experiment. Although it appears from the absolute values, shown in table 1, that the water increased, the differences do not show statistical significance.

DISCUSSION

The subject of overnutrition and its possible consequences on the life span are fairly well defined. Silberberg et al. ('55) reported a reduced life span in "yellow mice" fed enriched diets. More recently, Lane and Dickie ('57) demonstrated that restriction of caloric intake increased the life expectancy of genetically obese mice. Although neither of these authors reported on body composition patterns during the aging of their animals, this type of study has been performed. Yiengst et al. ('59) have shown that the muscle mass decreased with age and Conrad and Miller ('56) reported that the body fat doubled from 30 to 120 days. All studies on body composition patterns, however, have been made with animals that were fully grown or growing at a "normal" rate. The present study of body composition patterns has been made in rats that were retarded in growth yet had significantly increased life expectancies. It is significant to note that these animals had increased body protein and a reduction in the total body fat content; the rats were chronologically older but had been maintained at a constant body weight. From the foregoing, it would seem reasonable to conclude that in general there is a change in the chemical composition of the body, with special reference to the fat content, provided the animal is allowed to grow. If the animal is held to a constant body weight (a weight which can be attained in 10 days if the animal is fed ad libitum) by calorie restriction, however, the body fat, if anything, decreases with time. Although the body composition patterns have not been studied during the entire course of the life span, the 23-week point is well into the adult life of the rat and during this period chronologic aging in itself is not predisposing to fat deposition in the body.

SUMMARY

The body fat of hypocalorically retarded rats decreased from 4.90% at 13 weeks of age to 2.24% at 23 weeks of age. During

this time, the body protein had increased from 20.2 to 21.4%, and the body mineral had increased from 3.1 to 4.1%. By comparison, weight controls at 5 weeks of age had 6.39% of body fat, 17.1% of body protein, and 2.8% of minerals. Body water was noted to be the same for the weight controls and the retarded rats.

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kg	kilogram	km	kilometer
gm	gram	m	meter
mg	milligram	cm	centimeter
µg	microgram	mm	millimeter
mµg	millimicrogram	µ	micron
µµg	micromicrogram	mµ	millimicron
		µµ	micromicron

Volume		*Area*	
m^3	cubic meter		
cm^3	cubic centimeter		
mm^3	cubic millimeter	m^2	square meter
l	liter	cm^2	square centimeter
ml	milliliter	mm^2	square millimeter

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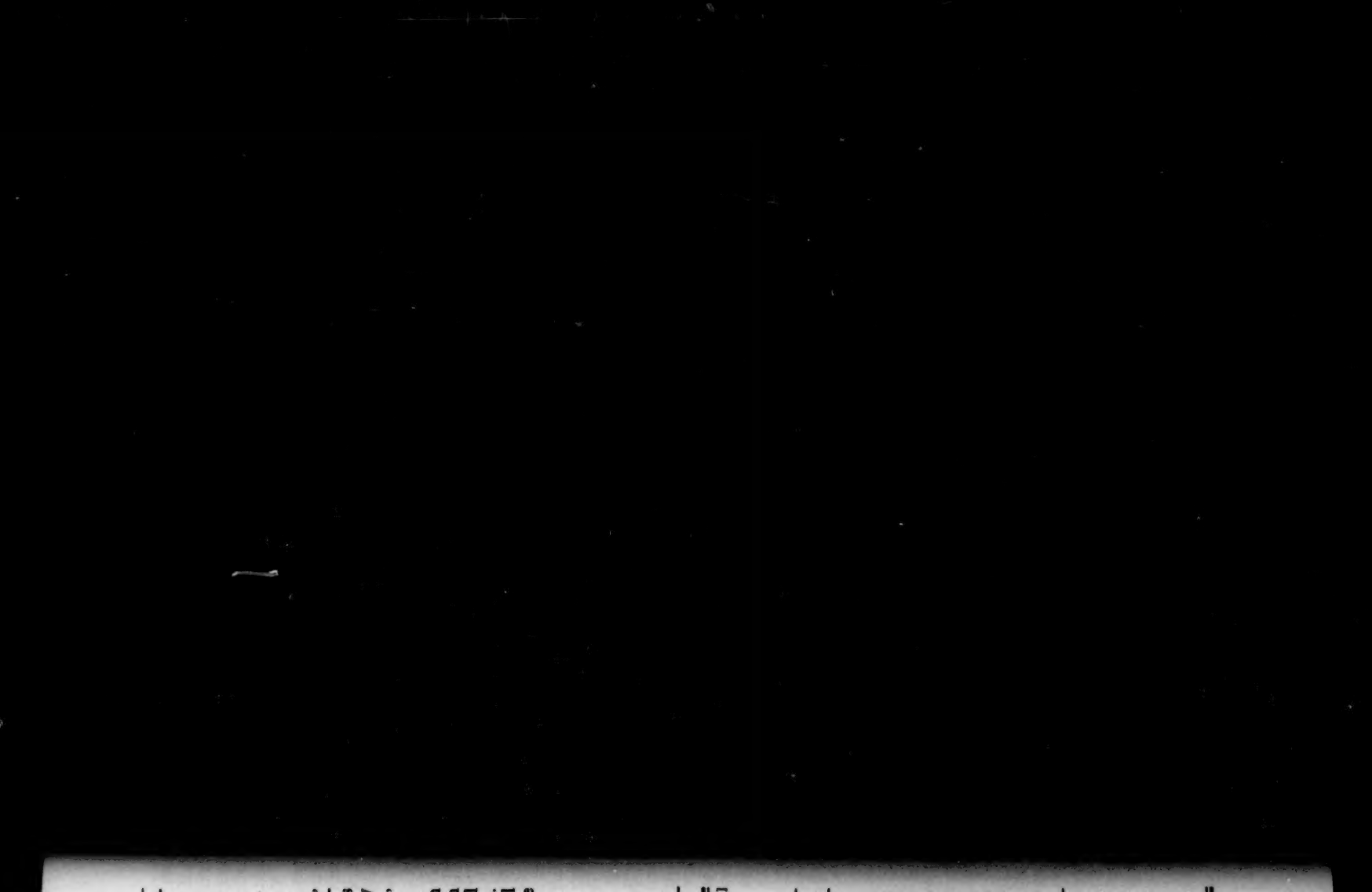
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